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VOL. 20, SEC. C. OCTOBER, 1942 NUMBER 10

RELATION OF LOOSE SMUT TO YIELD OF BARLEY[1]

BY WM. SEMENIUK[2] AND J. G. ROSS[3]

Abstract

A simple technique was devised to inoculate barley with *Ustilago nuda* (Jens.) K. and S. in order to facilitate the study of the effect of loose smut on yield. Infected seed lots so obtained were mixed in different proportions with normal seed to provide at least six levels of infection ranging from nearly zero to the maximum. The infection levels were planted in replicated field test plots at three stations in each of two years.

No significant correlations were obtained between yield of barley and the percentage loose smut in the 1939 trials. A direct linear relationship was obtained in 1940 between percentage incidence of loose smut and the reduction in yield. For every 1% increase in loose smut, yield was reduced 0.85% at St. Paul, Minn., 1.4% at Edmonton, and 1.2% at Fallis, Alta. These regression coefficients are not significantly different. Loose smut did not affect tillering noticeably. Similar infections of loose smut were obtained at all stations for comparable seed lots. Higher infections were observed as the rate of seeding was increased. The incidence of loose smut at Edmonton was the same on the basis of percentage smutted spikes as on the basis of percentage smutted plants.

Introduction

Loose smut of barley, caused by *Ustilago nuda* (Jens.) K. and S., is a very common disease in the field. Many fields are recorded showing 10% loose smut while a few fields have shown even 50% (1). The reduction in yield from this disease has not been determined but a high positive correlation has been assumed.

Previous attempts to determine the reduction in yield of barley caused by loose smut were rendered difficult through insufficient quantities of seed exhibiting high percentages of the fungus. Commercial seed samples proved unsatisfactory for this purpose because of their low percentages of loose smut and their likelihood of containing varietal mixtures. A simple technique for inoculating barley in the field with *U. nuda* was devised to provide adequate quantities of seed of known varietal purity, normal size and appearance, and which on planting exhibited a high percentage of loose smut. Such seed was mixed with different proportions of normal healthy seed to make samples showing different percentage levels of diseased seed. The samples so obtained were planted in replicated field trials at several stations from which the relationship of yield to the percentage of loose smut was determined.

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Experimental

Material and Methods

Newal barley (C.A.N. 1089*) obtained from certified seed stock of the University of Alberta, was the variety used in the present study. This variety had shown as high as 5% loose smut in commercial fields and averaged 35% over a three year period of artificial inoculation in field plots.

Method of Inoculation

Artificially infected seed lots of this variety were produced in Edmonton during the two years, 1938 and 1939, previous to their use in yield test plots. Inoculum of *U. nuda* consisted of chlamydospores obtained from a large composite collection of infected barley spikes. These spikes were collected during 1938 from the Uniform Barley Nursery and the rod row trials of the Edmonton station and from commercial barley fields in and around Edmonton whereas in 1939 they were collected from the loose smut yield trial at the Edmonton station. The spikes were spread out in the laboratory and allowed to dry overnight at room temperature after which they were ground with a meat chopper and passed through a 144 mesh fly screen. Sifted inoculum was then placed in a four layer cheese-cloth bag from which chlamydospores were dusted by hand over the developing barley heads. Inoculation of the barley flowers in the field began when the barley heads first emerged from the boot in early July. The chlamydospores of *U. nuda* were dusted heavily over each spike by jarring the bag of spores against each head. Such dustings were made each evening over a two week period until all but the very latest tillers had headed. In this procedure, each head was dusted each evening for the duration of the two week period, and in all, four quarts of chlamydospores were used during the season on approximately 80 sq. yd. of grain. At harvest time the youngest and, therefore, uninoculated spikes were pulled out and discarded. Ten pounds of infected Newal barley seed were obtained in 1938 and 20 lb. in 1939. Greenhouse tests of the infected seed lots produced in 1938 and 1939 revealed 15.5 and 33.0% loose smut, respectively.

Preparation of Seed for Yield Trials

Taylor (3) found that smaller wheat seed segregates show higher percentage of infection with *U. tritici* than larger seeds. Accordingly the bulk sample of infected barley produced in 1938 for the 1939 yield tests was separated into four grades of which only the intermediate size, weighing 35.0 gm. per 1000 kernels as compared to 39.2 gm. for the bulk lot, was used for the yield tests in 1939. The infected seed lot produced in 1939 weighed 37.5 gm. per 1000 kernels compared with 42.1 gm. for a similar non-infected seed lot. No separation of seed was made for the 1940 yield test. The infected seed lots were divided and "diluted" with normal non-infected seed of the same variety to obtain samples yielding different amounts of loose smut. Six levels of loose smut percentage were used at all stations in 1939, when the mean maximum percentage of loose smut in the crop was about 11%, whereas in 1940, when the mean maximum of loose smut was 32%, six and nine levels were used.

* *Canadian Accession Number.*

Design of Experiment

Yield trials were conducted in 1939 at Fallis, Castor, and Edmonton, Alta. (the latter station, however, producing no yield data owing to hail damage) and in 1940 at Edmonton and Fallis, Alta., and St. Paul, Minn. The soil types at these stations were as follows: black loam at Edmonton, gray wooded loam at Fallis (5), silt loam on the fringe of the dark brown soil zone at Castor (4), and Waukegan silt loam at St. Paul. A 6 × 6 Latin square arrangement was used at all stations in 1939 and at the two Alberta stations in 1940. A randomized block arrangement with six replications was used at St. Paul. The treatment rows of infected seeds were separated by a row of non-infected seeds. In every case the rows were 18 ft. long and one foot apart, except at Fallis and Edmonton in 1940, where the rows were seven inches apart. Fourteen grams of seed (or approximately 370 seeds) were drilled in each row in 1939, while 400 seeds were sown in each row in 1940.

Data were taken for each plot on the number of infected and non-infected tillers and yield in grams. In 1939 the infected spikes were clipped off and recorded as they emerged from the boot, but in 1940, they were left intact and recorded only after the plot was cut at harvest. Seed producing tillers only were considered in determining the number of non-infected tillers. Precautions were taken against loss of seed in handling the harvested rows by wrapping the sheaves in cloth and threshing by hand in a muslin bag.

Results

The 1939 Yield Trials

The results from the 1939 yield trials at Castor and Fallis are shown respectively in Tables I and II. In these tables and those that follow, only the means of the replications are presented because such means were found to yield the same general result in calculation of the regression of yield on percentage loose smut as did the values for the individual plots.

TABLE I

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND YIELD IN GRAMS OF SIX LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT FALLIS, ALTA., IN 1939

Treatment	Percentage loose smut	Total number of spikes*	Yield*, gm.
Infected seed	10.8	299	301
4 Parts infected/1 part non-infected	9.4	310	317
3 Parts infected /2 parts non-infected	5.8	303	314
2 Parts infected/3 parts non-infected	4.0	320	340
1 Part infected/4 parts non-infected	2.3	309	322
Non-infected seed	0.1	327	337

** F value obtained for 5 D.f. for treatment and 20 D.f. for error is 0.78 for total number of spikes and 1.5 for yield, both being non-significant.*

TABLE II

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND YIELD IN GRAMS OF SIX LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT CASTOR, ALTA., IN 1939

Treatment	Percentage loose smut	Total number of spikes*	Yield*, gm.
Infected seed	11.5	376	353
4 Parts infected/1 part non-infected	8.9	394	376
3 Parts infected/2 parts non-infected	6.1	416	380
2 Parts infected/3 parts non-infected	4.6	398	377
1 Part infected/4 parts non-infected	1.5	414	382
Non-infected seed	0.2	438	427

* *F value obtained for 5 D.f. for treatment and 20 D.f. for error is 0.91 for total number of spikes and 1.9 for yield, both being non-significant.*

No statistically significant regressions for yield on the percentage loose smut were demonstrable among the data obtained at Fallis and Castor. The lowest yield was found in the treatment showing the mean maximum of smut infection at both stations. This tendency for a relationship suggested the repetition of the trials in 1940.

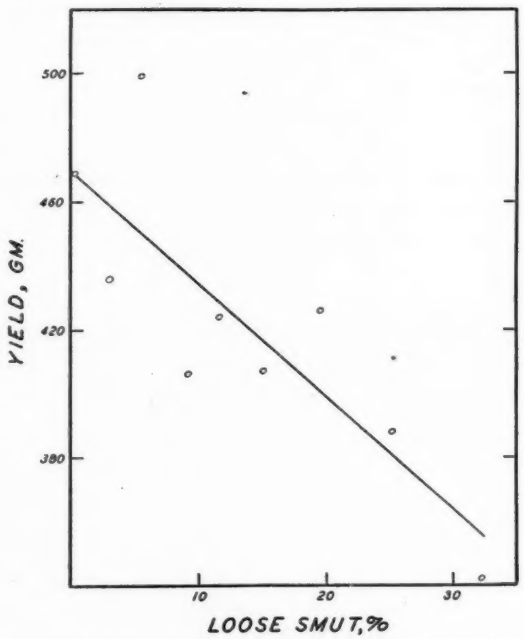

FIG. 1. *Regression of yield on percentage loose smut in Newal barley grown at St. Paul, Minn., in 1940.*

The 1940 Yield Trials

Yield trials in 1940 were conducted at three stations: St. Paul, Minn.; Edmonton and Fallis, Alta.

St. Paul, Minn.

The results obtained at St. Paul, with nine levels of loose smut, are presented in Table III and the regression of yield on percentage loose smut is graphically represented in Fig. 1. The maximum mean percentage of loose smut at this station was 32.5 and the minimum 0.3. Highly significant differences for treatment yields were obtained. The minimum yield of 342 gm. occurred with the treatment in the highest infection level, namely, 32.5% loose smut, and the maximum yield of 499 gm. occurred in the treatment showing 5.5% loose smut. The regression of yield per plot on percentage loose smut was highly significant (P less than 0.001) with a 1% increment of loose smut reducing yield 0.85% or 3.6 gm.

TABLE III

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND YIELD IN GRAMS OF NINE LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT ST. PAUL, MINN., IN 1940

Treatment	Percentage loose smut	Total number of spikes	Yield, gm.
Infected seed	32.5	514	342
8 Parts infected/2 parts non-infected	25.2	523	388
6 Parts infected/4 parts non-infected	19.5	508	426
5 Parts infected/5 parts non-infected	15.1	489	407
4 Parts infected/6 parts non-infected	11.6	489	424
3 Parts infected/7 parts non-infected	9.1	529	406
2 Parts infected/8 parts non-infected	5.5	494	499
1 Part infected/9 parts non-infected	3.1	486	436
Non-infected seed	0.3	488	469

Difference necessary for significance at the 5% point = 50 spikes, 58.8 gm.

Edmonton, Alta.

The data for Edmonton are presented in Table IV and the regression of yield on percentage loose smut is shown in Fig. 2. At this station only six percentage levels of infection were included in the yield test with the maximum amount of loose smut at 30.5% as a treatment mean and the minimum at 0.2%. The yield varied correspondingly from 280 gm. at the mean minimum to 427 gm. as the mean maximum. Highly significant differences in yield were obtained with the six percentage levels of loose smut. The regression of yield on percentage loose smut was highly significant (P less than 0.001) with a 1% increment of loose smut reducing yield 1.4% or 4.8 gm.

Fallis, Alta.

The data for this station are summarized in Table V and the regression of yield on percentage loose smut is indicated in Fig. 3. Six percentage levels of loose smut were included in this yield test as at Edmonton. Samples from

TABLE IV

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND THE YIELD IN GRAMS OF SIX LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT EDMONTON, ALTA., IN 1940

Treatment	Percentage loose smut	Total number of spikes	Yield, gm.
Infected seed	30.5	361	280
4 Parts infected/1 part non-infected	24.8	359	295
3 Parts infected/2 parts non-infected	16.5	370	341
2 Parts infected/3 parts non-infected	11.1	359	341
1 Part infected/4 parts non-infected	6.8	381	394
Non-infected seed	0.2	396	427

Difference necessary for significance at the 5% point = 28 spikes, 32.1 gm.

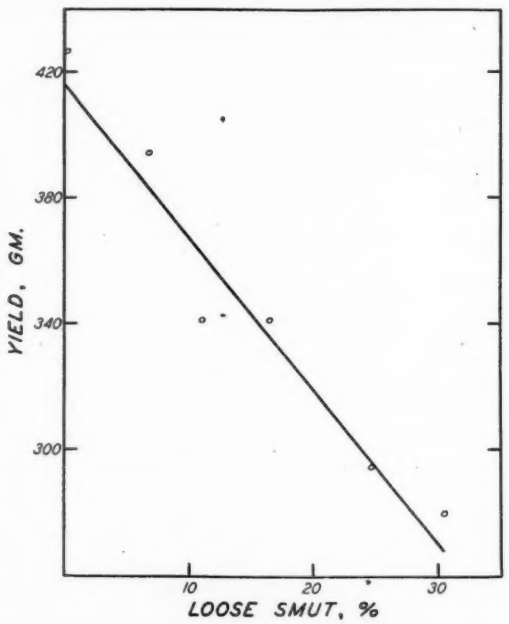

FIG. 2. *Regression of yield on percentage loose smut in Newal barley grown at Edmonton, Alta., in 1940.*

the same infected seed lots were used at both stations. At the Fallis station the maximum occurrence of loose smut was 32.4% as a treatment mean and the minimum 0.5%. The yield varied correspondingly from 141 to 207 gm. The variance in yield at this station was the lowest of the three stations in 1940. Highly significant differences in yield were obtained with the six different levels of loose smut. The regression of yield on percentage of the disease was highly significant (P less than 0.001). A 1% increment of loose smut reduced yield 1.2% or 2.1 gm.

TABLE V

MEANS OF SIX REPLICATES FOR PERCENTAGE LOOSE SMUT, TOTAL NUMBER OF SPIKES, AND YIELD IN GRAMS OF SIX LEVELS OF INFECTION WITH *Ustilago nuda* ON NEWAL BARLEY GROWN AT FALLIS, ALTA., IN 1940

Treatment	Percentage loose smut	Total number of spikes	Yield, gm.
Infected seed	32.4	297	141
4 Parts infected/1 part non-infected	24.8	315	156
3 Parts infected/2 parts non-infected	18.4	314	166
2 Parts infected/3 parts non-infected	13.0	315	178
1 Part infected/4 parts non-infected	5.5	304	198
Non-infected seed	0.5	322	207

Difference necessary for significance at the 5% point = 49 spikes, 11.0 gm.

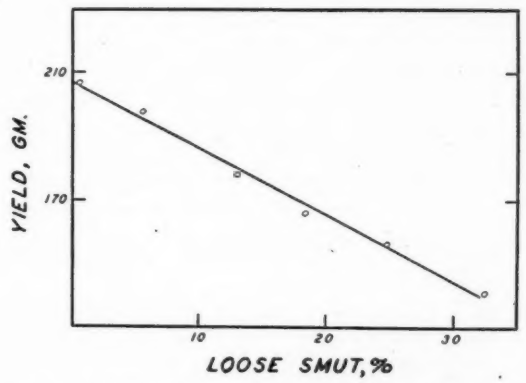

FIG. 3. *Regression of yield on percentage loose smut in Newal barley grown at Fallis, Alta., in 1940.*

Influence of Rate of Seeding of Infected Barley Seed on Yield

One difference in the loose smut yield test trials conducted in 1939 and in 1940 was in the rate of seeding. In 1939, 14 gm. of seed, approximately 370 seeds, were drilled in each 18 ft. row while in 1940, 400 seeds were planted in rows of the same length. A yield test was therefore conducted in St. Paul in 1940, in a randomized block with four replications; infected and non-infected seed was used to determine the influence that rate of seeding had on the incidence of loose smut and the consequent reductions in yield. The rates of seeding were: 108 seeds, 216 seeds, and 648 seeds per row. Each plot contained three rows 18 ft. long and one foot apart; only the centre row was harvested. The results obtained are summarized in Table VI. Significantly higher percentages of loose smut occurred at the higher rates of seeding. Seeds artificially infected with *U. nuda* showed 26.9, 30.1, and 34.0% loose smut, respectively, for seeding rates of 108, 216, and 648 seeds per plot. Non-infected seed showed 0.0, 1.2, and 0.8% loose smut, respectively, at

TABLE VI

RATE OF SEEDING AND INCIDENCE OF LOOSE SMUT ON YIELD OF NEWAL BARLEY AT ST. PAUL, MINN., 1940

Rate of seeding, No. seeds per row	Seed used	Percentage infection	Total number of spikes	Mean number of spikes per seed sown	Yield, gm.
108	Infected	26.9	334	4.0	329
	Non-infected	0.0	335	4.0	442
216	Infected	30.1	432	2.6	330
	Non-infected	1.2	414	2.5	480
648	Infected	34.0	587	1.2	355
	Non-infected	0.8	509	1.0	498

these same rates of seeding. Yield per plot from non-infected seed was significantly higher at all rates of seeding than the yields from the infected seed.

No statistically significant differences in yield were found between the three rates of seeding within either the infected or non-infected seed lots. The very similar yields obtained in this test suggest that small variations in the amount or number of seeds sown per row do not affect yield to any appreciable amount.

Incidence of Loose Smut Compared on the Basis of Total Number of Plants and of Total Number of Spikes

The method used in the present study of recording the incidence of loose smut on the basis of total number of infected and non-infected spikes was compared with the percentage of plants showing loose smut. For such a comparison, a side experiment was conducted at Edmonton in 1939 and 1940. Samples from the same seed mixture as used in the regular yield trials were obtained for the experiment to determine the percentage of smutted plants at the different levels of infection. Fifty seeds were spaced with a "V" belt seeder in an 8 ft. row in 1939 and 150 seeds were spaced in an 18 ft. row in 1940. This test was conducted in a quadruplicated randomized block arrangement. The percentage of plants showing loose smut was determined in early fall by pulling up the entire row of plants and recording the number of infected and non-infected plants. The results obtained are summarized in Table VII, together with the percentage of spikes showing loose smut as obtained from the regular yield trial.

In general, percentage values obtained for loose smut on the basis of number of plants infected were similar to those obtained on the basis of number of spikes infected. Close agreement was also obtained with the data from the other two stations at Fallis and Castor in 1939, and at Fallis and St. Paul. The fairly uniform results obtained in the incidence of loose smut at three widely different stations in 1939 and again in 1940 suggest that soil and climatic conditions have only a minor influence on the expression of this disease.

TABLE VII

PERCENTAGE LOOSE SMUT AS COMPARED ON THE PER PLANT AND THE PER SPIKE BASIS, AT COMPARABLE INFECTION LEVELS

1939				1940			
Spike basis			Plant basis	Spike basis			Plant basis
Fallis	Castor	Edmonton*	Edmonton	Fallis	Edmonton	St. Paul	Edmonton
10.8	11.5	8.7	9.0	32.4	30.5	32.5	35.8
9.4	8.9	6.6	4.2	24.8	25.8	25.2	25.4
5.8	6.1	4.9	4.2	18.4	16.5	19.5	20.6
4.0	4.6	4.5	3.7	—	—	15.1	—
2.3	1.5	2.3	3.4	13.0	11.1	11.6	12.4
0.1	0.2	0.0	0.0	—	—	9.1	—
				5.5	6.8	5.5	5.3
				—	—	3.1	—
				0.5	0.2	0.3	1.4

* *Because of a damaging hail-storm, yield data were not obtained at this station.*

Discussion

Loose smut of barley follows from a systemic infection of the barley plant by *U. nuda*. Although the influence of such an infection on tillering was not determined on the per plant basis in the present study, the results obtained in the regular yield trials indicated that tillering of plants, as determined by the number of spikes formed, was not affected in the early stages of seedling growth. The general agreement of values for percentage of infected plants with the percentage of infected spikes pointed to the occurrence of smut on all spikes produced by an infected plant. Support for this observation was found in the recent work of Oort (2), who noted that from 2290 plants infected with *U. nuda*, 96.2% showed all heads completely destroyed or, at most, not over one head of a plant less than one-half healthy. In only very few instances were partially diseased spikes encountered in the tests reported in this paper.

No satisfactory explanation can be offered for the lack of significant differences in yield between the different percentage levels of loose smut in 1939. Small variations in the number of seeds sown had little effect on the yield of grain when either infected or non-infected seed lots were used.

The differences between stations during 1940 in the percentage decreases in yield for every 1% increase in loose smut are not real. The regression coefficient of 0.85% at St. Paul is not significantly lower than the coefficients of 1.4% and 1.2% for Edmonton and Fallis, respectively.

Acknowledgments

The authors are indebted to Dr. K. W. Neatby, formerly Professor of Field Crops, University of Alberta, for suggesting this study and for his advice in the preparation of the manuscript. Dr. F. R. Immer, Professor of Agronomy

and Plant Genetics, University of Minnesota, willingly advised in the analysis and the interpretation of the data. His help is gratefully acknowledged. They wish to thank Dr. H. K. Hayes, Chief of the Division of Agronomy and Plant Genetics, University of Minnesota, for permission to carry on the test, and for field assistance, at the University Farm, St. Paul, Minn.

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HOST–PARASITE RELATIONSHIPS IN A SEED-BORNE DISEASE OF BARLEY CAUSED BY *HELMINTHOSPORIUM SATIVUM* PAMMEL, KING, AND BAKKE[1]

By H. W. Mead[2]

Abstract

A hulled barley kernel is a caryopsis enclosed by the tightly adhering floral glumes. The testa, which is formed from the inner integument of the ovule, becomes increasingly resistant to fungi. Spikelets may become parasitized during flowering and afterwards, by air-borne spores and fragments of mycelium of *H. sativum*. The fungus may cause blighting, shrivelling, and discoloration of the spikelets and maturing kernels, the damage depending upon the time of inoculation. Irregular, dark, thick-walled mycelium may be found massed in the parenchyma of the glumes, pericarp, and lodicules, and ungerminated spores between the glumes and pericarp. This dormant mycelium will remain viable for from two to five years. It germinates when the kernel germinates and infects the young tissues of the plumule and radicle as these organs expand and force their way through and past the infected tissues. It also colonizes the soil nearby, to a limited extent. Embryo blight, pre-emergence and postemergence blight, with malformation, stunting, and lesioning of the seedling may follow infection, the amount of each depending upon certain environmental factors.

Introduction

Many seed samples of barley received from various districts in Canada contain kernels that are infected with the fungus *Helminthosporium sativum* Pammel, King, and Bakke. This fungus may become established in the kernel while it is developing and it may infect various parts of the embryo during germination. The purpose of this study was to determine the relation of the fungus to various parts of the kernel and developing embryo.

The disease on the kernel usually is indicated by a diffuse pale to dark brown discoloration of the lemma and palea, especially in the region of the embryo and groove (Fig. 1, A, B). This staining may spread over the dorsal surface of the kernel or occur as isolated brown spots, or at times the fungus may be present without any symptoms being evident. Infected kernels may be shrivelled, but they usually are as plump or plumper than healthy kernels. The terms smudge, kernel smudge, and black point have been used to describe the affected kernels, but these terms have been used also to describe similar discolorations caused by other fungi. The term smudge is being used in this paper.

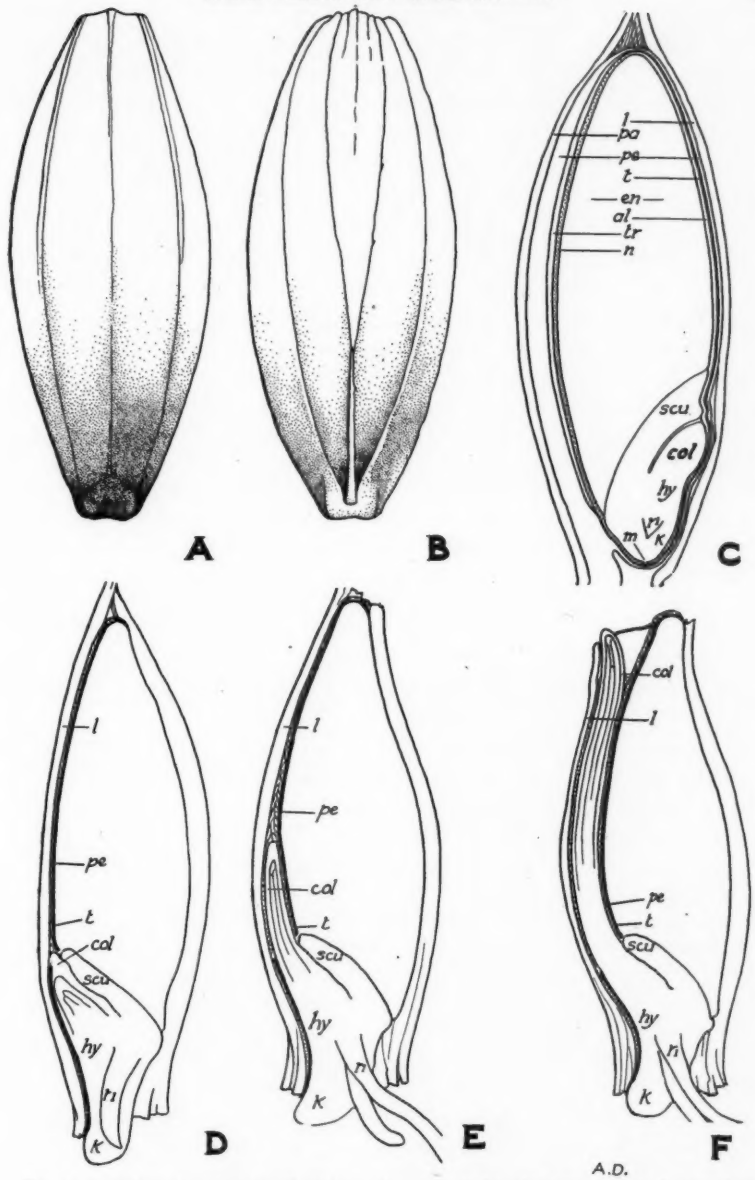
The disease on the seedling may take the following forms: embryo blight, pre-emergence and postemergence blight, with malformation, stunting, and lesioning of the seedling. The plumule and roots may be rotted before they emerge from the hulls of the kernel, or later they may become distorted and

[1] *Manuscript received in original form January 26, 1942, and as revised, July 10, 1942. Contribution No. 712 from the Division of Botany and Plant Pathology, Science Service, Dominion Department of Agriculture, Ottawa, Canada. Based on part of a thesis submitted to the University of Toronto in 1942 in partial fulfilment of the requirements for the degree of Doctor of Philosophy.*

[2] *Assistant Plant Pathologist.*

weak, and unable to penetrate the soil. The majority of the seedlings emerge from the soil. Of these, a few may remain in a stunted condition for a time and then die. The rest may develop in an apparently normal manner but most of them bear brown, circular to oblong lesions on the roots and coleoptile and occasionally on the leaves.

This characteristic discoloration of barley kernels has been observed and described in Europe and North America. Zöbl (23) called it "Braunspitzige", and concluded that *Cladosporium herbarum* (Pers.) Lk. caused the discoloration. Puchner (15) called it "Schwartzspitzige" and found that discoloured kernels germinated poorly and after germination often produced seedlings with lesions on the leaves. Ravn (17) also used the term "Braunspitzige", but attributed it to species of *Helminthosporium* of which *H. teres* Sacc. was found to occur frequently in cultures of discoloured barley kernels. Peyronel's (14) "Puntatura" appears to refer to the same malady. He said that the integuments of the kernel became discoloured owing to the presence of a brown pigment, and that although the pigment was confined chiefly to the layer of horizontal cells overlying the epicarp, it sometimes occurred in the epicarp as well. The cells of the aleurone layer often were found to be discoloured but not those of the embryo. The first symptom of the disease was a discoloration of the lodicules which are close to the scutellum. At the base of these there was found a thin-walled tissue rich in nutrients, which collapsed early. This was considered to be a likely substratum from which the fungus might invade the integuments overlying the embryo. He showed that *C. herbarum* was sometimes associated with the discoloration, but not the cause of it. Rosella (18) found that *Helminthosporium gramineum* (Rab.) Erik. caused a seed discoloration in barley which he called "Moucheture". He observed also that *H. sativum* invaded barley spikes while they were still enclosed by the leaf sheaths. The fungus penetrated the sheaths and young glumes and invaded the kernels. Christensen and Stakman (2) described "seed blight" in barley in which heavily infected seeds were discoloured, shrivelled, and of inferior quality and from which seedling blight, root rot, basal stem rot, and decrease in yield often resulted. They found species of *Fusarium*, *Helminthosporium*, and *Alternaria* to be the fungi most commonly present. Güssow (9) reported a seed-borne disease of barley that was caused by *H. sativum*. It caused spotting and shrivelling of the grain and leaves. Machacek and Greaney (12) described a similar discoloration in the seeds of cereals, which was referred to as black point or kernel smudge. They considered that it was caused mostly by fungi, the predominating forms being *Alternaria* spp., *H. sativum*, *H. teres*, and *Fusarium* spp. In certain years, one of these fungi was much more common than the others on cereal seeds. For instance, in 1935, *H. sativum* was predominant, and in the period 1931 to 1934, two species of *Alternaria* were predominant. Dickson (5) described the condition under the heading, spot blotch of barley, *Helminthosporium sativum* P.K. & B. The discoloured and sometimes shrunken kernels were described as "blight damaged" and it was pointed out that they are given the



A.D.

FIG. 1. *Surface views and longitudinal sections of hulled barley kernels. A, B, dorsal and ventral surfaces respectively of smudge kernels, showing by means of shading, the location of the typical discoloration; C, median longitudinal section showing the extent and relation of the parts of a dormant kernel; D, E, F, longitudinal sections showing the relation of the parts of germinating kernels, 2.5, 3.5, and 5.5 days, respectively, after being placed in germinators at 10° C. Al, aleurone; col, coleoptile; en, endosperm; hy, hypocotyl; k, coleorhiza; l, lemma; m, micropyle; n, nucellus; pa, palea; pe, pericarp; r, primary root; scu, scutellum; t, testa; tr, transchalazal tissue. ×9 approx.*

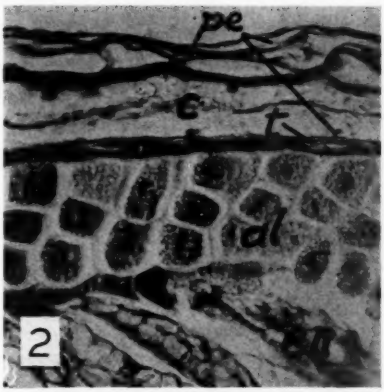
special grade designation "blighted" in the Federal Grain grades of United States. The disease is common in the southern part of the barley zone but is considered to be of minor importance. In Nebraska, in 1941, this disease was common, many samples of barley containing as high as 38% blighted kernels (8). In Canada, the most severely infected samples have been obtained from New Brunswick and Manitoba.

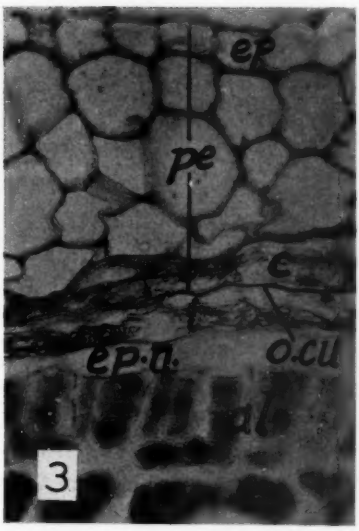
The Development and Structure of a Healthy Hulled Barley Kernel

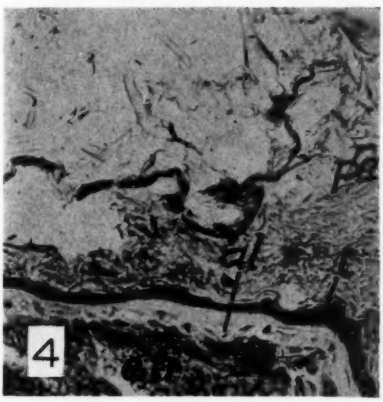
A hulled barley kernel is a caryopsis closely invested by the floral glumes (Fig. 1C, *l*, *pa*). These glumes, lemma and palea, begin to adhere to the pericarp (ovary wall) about 20 and 10 days, respectively, after flowering (10). They consist of thick-walled ground tissue, vascular strands, and longitudinal green bands of thin-walled chlorophyllous parenchyma. The outer epidermis consists of alternating large and small cells with thick walls. The walls have characteristic wavy thickening and simple pits. The cells of the inner epidermis have thinner walls. There are stomata on both inner and outer surfaces. Both epidermises become cutinized and silicated and when mature are very hard in texture. The outer glume, the lemma, overlaps the palea, and together they provide a hard protective covering for the caryopsis. When young, they are soft in texture and may become infected with fungous mycelium which also may grow over the edges and ends to reach the developing ovary. Spores of fungi also may lodge inside the glumes before the glumes begin to adhere to the pericarp.

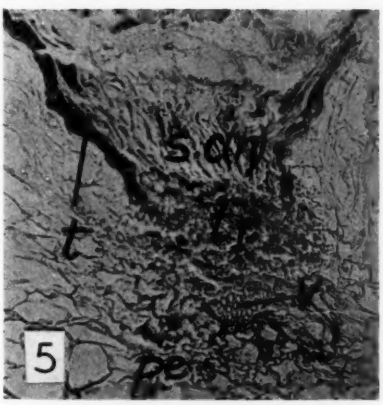
The pericarp (the ovary wall) of the young caryopsis consists of several layers of parenchyma cells, arranged in two main layers. The inner, cross layer, is chlorophyllous and its cells are thick-walled (Figs. 2 and 3, *c*). There are inner and outer epidermal layers and these are thinly cutinized even at flowering time. They bear stomata and hairs. By the end of the first week after flowering, the inner epidermis has been absorbed and the pericarp itself is being absorbed (21) (Fig. 2, *pe*). Absorption of the pericarp is complete by the end of the second week and there is left a much flattened outer epidermis with a cuticle, covering a few rows of collapsed parenchymatous cells. This cuticle adheres to that of the glumes. The pericarp encloses the caryopsis completely (Fig. 1C, *pe*). It is thin over the embryo; in the region of the furrow it is thick and contains a vascular trace which extends along the length of the caryopsis (Figs. 5 and 6, *v*).

The inner integument of the ovule (Figs. 3 and 6, *i*), consisting of two layers of cells, eventually forms the testa of the mature caryopsis (Figs. 2, 4, and 5, *t*). As a result of compression and absorption it forms a narrow homogeneous, brown, oily-appearing layer bordered on both sides by cutin membranes, the outer being much thicker than the inner. This outer membrane increases in thickness from flowering to maturity. The testa completely envelops the caryopsis (Fig. 1C, *t*) except over the micropyle and in the

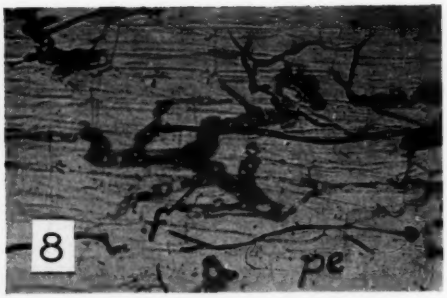


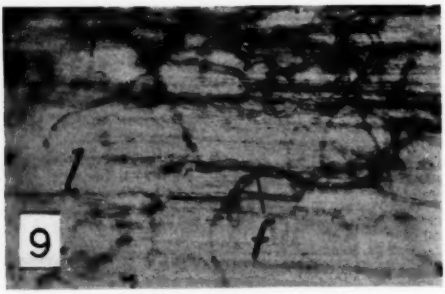


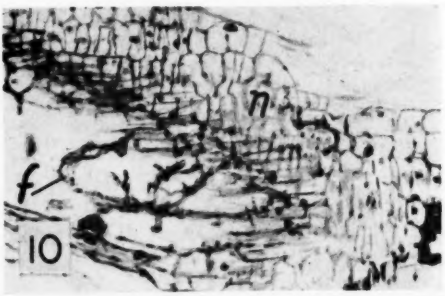


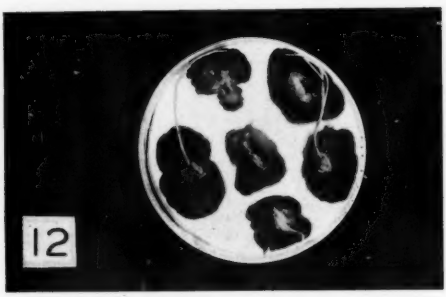


FIGS. 2 TO 6. *Cross and longitudinal sections of normal barley kernels, stained with erythrosin and malachite green. Al = aleurone; c = chlorophyllous (cross) layer of pericarp; en = endosperm; ep = epidermis of pericarp; ep.n = epidermis of nucellus; i = inner integument; o.cu = outer cuticle of inner integument; pe = pericarp; s.c(n) = sheaf cells (nucellus); t = testa; tr = transchalazal tissue; v = vascular trace of pericarp.* FIG. 2. *Cross section of dorsal surface of kernel, almost mature. ×375.* FIG. 3. *Dorsal surface of kernel seven days after flowering. ×375.* FIG. 4. *Longitudinal section of apex of kernel, almost mature. ×180.* FIG. 5. *Region of furrow almost mature. ×200.* FIG. 6. *Cross section of region of furrow, seven days after flowering. ×200.*









FIGS. 7 AND 8. *Strips of the pericarp of the kernels naturally infected with H. sativum showing dormant mycelium of the fungus (f). ×350.* FIG. 9. *Mycelium in the lemma. ×200. Stained with acid fuchsin.* FIG. 10. *A longitudinal section showing mycelium (f) of H. sativum in the embryo sac of a spikelet inoculated at flowering time. ×200. Stained with erythrosin and malachite green.* FIG. 11. *H. sativum on the surface of a naturally infected kernel after five days in a moist chamber at 24° C. ×15.* FIG. 12. *Colonies of H. sativum from infected kernels on potato dextrose agar for seven days. ×1/3.*

region of the groove where it originates sharply at either side of the vascular trace (Figs. 5 and 6). Between the flanks of the testa is a strand of chalazal cells, the base of the ovule (Figs. 5 and 6, *tr*). The chalazal cells become thick-walled, suberized, and filled with fatty deposit (21). This is evident by the 12th day after flowering. It has been suggested by Krauss (11) that in some varieties of barley the outer cuticle of the integument and at times the inner cuticle may be absent from the micropylar area. Tharp (21) found them to be much thinner in that region than elsewhere. These cutin membranes are thick over the apex of the caryopsis (Fig. 4, *t*) and in the flanks of the furrow. The entire testa (integument plus membranes) varies in thickness. It is thin over the dorsal and lateral surfaces, distinctly thin over the embryo, thick over the apex of the kernel and at the flanks of the furrow.

The nucellus of the ovule becomes absorbed by the developing endosperm and is represented in the mature kernel by a structureless layer inside the testa over the dorsal surface and flanks, and a group of glandular cells in the region of the furrow (Figs. 5 and 6, *s.c*(*n*)). This group of cells, called "sheaf cells" by Tharp (21) and Collins (3) is thought to be part of the nucellus and serves to distribute supplies to the endosperm and embryo from the vascular trace of the pericarp. In wheat it is considered to be a barrier to fungous invasion because of its thickness and chemical composition (16).

The aleurone layer is a complete envelope except at the micropyle, where it is discontinuous (Fig. 1C, *al*). It consists of a single layer of elongate cells over the embryo and three or four layers of rectangular cells over the face and flanks of the kernel (Figs. 2 and 3, *al*). In the region of the furrow it has an irregular structure. At maturity this layer contains storage protein.

The endosperm develops by absorption of the nucellus. At maturity its contents are relatively dry and consist of hexose compounds. This condition is thought to be a barrier to extensive fungus invasion of the endosperm of wheat (16).

The embryo consists of a short hypocotyl, at the apex of which is the plumule enclosed by the coleoptile (Fig. 1C, *col*) and at the base the radicle consisting of the root initials enclosed by the coleorhiza (Fig. 1C, *k*). Attached to one side of the axis and forming the greater portion of the embryo is the scutellum (Fig. 1C, *scu*). Percival (13) found that there were small globules of a fatty oil and granules of protein in the parenchyma of the scutellum and other parts of the embryo of wheat. Soon after germination starts, starch appears in the coleorhiza, root cap, and tip of the coleoptile, derived from fat and a soluble carbohydrate stored in the tissues of the embryo.

From the preceding description, it appears that the barley spikelet would be most susceptible to fungus invasion at flowering time and during the week following that period. During this time its defences are rather weak and some of its tissues provide a good substratum for the fungus. The cutin membranes of the pericarp and testa are present at flowering time but are thin. The glumes are soft at first and have not become sealed to the pericarp and the lodicules are distended for a time with nutrient materials. These young

tissues are readily parasitized by *H. sativum* and they provide nourishment for the fungus. There are certain structural weaknesses at this time. The areas around the micropyle and over the embryo are thinly covered and the chalazal tissue at the flanks of the testa has not developed its suberin lining. However, these weaknesses soon disappear and the kernel becomes enclosed in a resistant envelope, reinforced by hard glumes which adhere tightly. Similarly, the nutrient substances available to the fungus in the early stages disappear and the tissues which supplied them, collapse. These changes place a limit on the period during which air-borne fungi can successfully infect the kernel.

Chemical Changes during Development of the Spike

The writer has demonstrated the presence of reducing sugars in the parenchyma of the palea and lemma of young barley kernels two to three days after flowering, and again at 10 and 12 days after flowering. There were reducing sugars present also in the chlorophyll layer of the pericarp of 10-day old kernels. Tharp (21) reports a gradual change in the nature of the cell walls of the inner integument after the fifth day. The soft cellulose becomes less easily hydrated and thickens. At the same time the outer cutin membrane increases in thickness. At this time there are disorganized masses of endosperm and nucellar tissue in the regions of the embryo and chalaza. These are composed partly of much-hydrated cellulose and give a positive test for mucilage. The cells of the endosperm are filling with starch, and protein grains form in the aleurone during the second week after pollination; fat deposits are present in the mature kernel but at that time are very resistant to fat solvents. Eckerson (7) studied the chemical development of wheat from the primordia of the flower to the mature grain. Her findings with respect to the period following heading, i.e., the period during which the spike is exposed to wind-borne spores, are as follows. The pericarp breaks down rapidly and its contents, amino acids, glucose, fructose, and starch, move into the developing embryo sac to the embryo via the chalazal tract and nucellus. This movement is rapid at the time wall formation in the endosperm is beginning and continues during the development of the kernel. The nucellus soon breaks down, with only the outer membrane remaining. At the time morphological development of the endosperm is complete, a stream of sugar and amino acids is passing from the rachis through the chalaza and endosperm to the embryo. Starch begins to form in the endosperm soon after wall formation and it continues to increase in amount until desiccation begins. There are amino acids and a small amount of fat in the endosperm also. The aleurone layer at this time contains magnesium, phosphate, and calcium with storage protein appearing later. The latter, with gluten, appears in the endosperm only at the time desiccation begins. At flowering time, the stigma contains much pectin and a little glucose. These substances are exuded and also are present all the way to the embryo sac.

Histological Studies of Barley Kernels Infected with *H. sativum*

A. Materials and Methods

A diseased sample of Charlottetown No. 80 barley from New Brunswick, and a sample of Colsess barley from Manitoba were used in this study. The disease on the seed was studied by means of stained strips of the seed coverings and by microtome sections. Thin strips of glumes, pericarp, and root and shoot tissue were boiled in lactophenol containing cotton blue, or acid fuchsin and were mounted in lactophenol. Diseased mature kernels were soaked in water, the hard palea and lemma removed, and the remainder fixed in formalin acetic alcohol (1). Kernels in various stages of development, inoculated with the fungus *H. sativum*, also were fixed. Butyl alcohol was used for dehydration. Serial sections 10 to 15 μ in thickness were stained with a saturated aqueous solution of erythrosin and a weak solution of malachite green in absolute alcohol. The early stages of seedling infection during germination were studied by means of strips and microtome sections also. Solubility and staining tests for membrane substances were applied to hand sections of the coleoptile as follows: pectin, soluble in hot water; pectic acid, soluble in warm two per cent potassium hydroxide or ammonia; calcium pectate, soluble in hot potassium hydroxide after heating in two per cent hydrochloric acid; cutin, stains yellow or brown in chloro-iodide of zinc.

B. Examination of Field Material

1. Development in Culture

In order to establish the presence of *H. sativum* in the two seed samples of barley that were thought to be infected with the fungus, representative lots were plated on potato dextrose agar and on moist filter paper (Figs. 12 and 11). The New Brunswick sample contained many kernels of the smudge type and the rest of the kernels had a weathered appearance. Some of this seed was surface sterilized by immersing it for three minutes in a mixture containing one part of 95% alcohol and three parts of 1 : 1000 aqueous solution of mercuric chloride, and then rinsing it in sterile water. Kernels taken at random from the surface sterilized lot, were plated on moist filter paper and incubated at 24.5° C. for four days. *H. sativum* developed on all of the kernels. The Manitoba sample was tested four times on potato dextrose agar and on moist filter paper. *H. sativum* grew from over 95% of the kernels in each test.

2. Occurrence of Mycelium in the Coverings of Naturally Infected Kernels

An examination of the tissues of kernels from these samples was undertaken to determine the location of the fungus. For this purpose the mature kernels were soaked in formalin acetic alcohol overnight and then strips of the lemma, palea, and pericarp were removed. These strips were stained and examined with a microscope. The results are given in Table I.

TABLE I

Helminthosporium sativum IN TISSUES OF MATURE BARLEY KERNELS

Tissue	Occurrence of mycelium in strips from:								
	Proximal end		Centre		Distal end		Both ends	Entire surface	Total
	A	B	A	B	A	B	A	A	A
	%		%		%		%	%	%
Lemma	12	++++	3	+	21	+++	0	24	60
Palea	26	++++	8	++	26	+++	3	13	76
Dorsal pericarp									
Outer layer	17	++++	8	+++	13	+++	13	4	55
Inner layer	20	+	10	++	11	++	12	6	59
Ventral pericarp									
Outer layer	11	+++	2	+	4	+++	0	11	28
Inner layer	0	0	0	0	0	0	0	0	0

NOTE: *A* = *Strips containing mycelium.*
B = *Prevalence of mycelium.*

The results show that mycelium was present to some extent in all of the seed coverings except in the innermost layer of the pericarp on the ventral side of the kernels. It was not found in every strip, nor was it co-extensive with them. It was present more commonly towards the ends of the kernels, particularly the proximal end, where most of the discoloration occurs. The depth of penetration by the fungus is indicated by the presence of hyphae in the inner layer of the pericarp on the face of the kernel. Hand sections failed to show deeper penetration. No mycelium was found in the embryo of mature kernels. It will be shown later that infection of the embryo caused blighting and shrivelling and such kernels are blown away during cleaning.

The mycelium on the surface of the cells was, for the most part, dark, thick-walled, and highly septate, and it contained protoplasm. Acid fuchsin stained the protoplasm deeply and left the walls very lightly stained. The strands appeared in various forms, some being short and grouped to form plates, or ladder-like formations (22), others being long and branched. Single strands containing only a few cells were found also (Figs. 7 and 8, *f*).

This mycelium was found to be on the surface, between and within the cells of the various seed coverings. There was a tendency for the strands of mycelium on the outer pericarp to be almost entirely on the surface of its outer epidermis, although some penetration was evident. The mycelium in the glumes had penetrated the thin walls of the parenchyma and could be found in the cells of the inner epidermis (Fig. 9). In the inner or cross layer of the pericarp, the hyphae were found to be both inter- and intracellular.

Points of penetration of the outer epidermis of the pericarp were observed. These points were indicated in the tissue when it was stained with acid fuchsin, by red areas on the otherwise lightly stained walls. In the centre of such an area there appeared a small translucent spot. This was the actual point of penetration. The remainder of the area, in which the colour became more

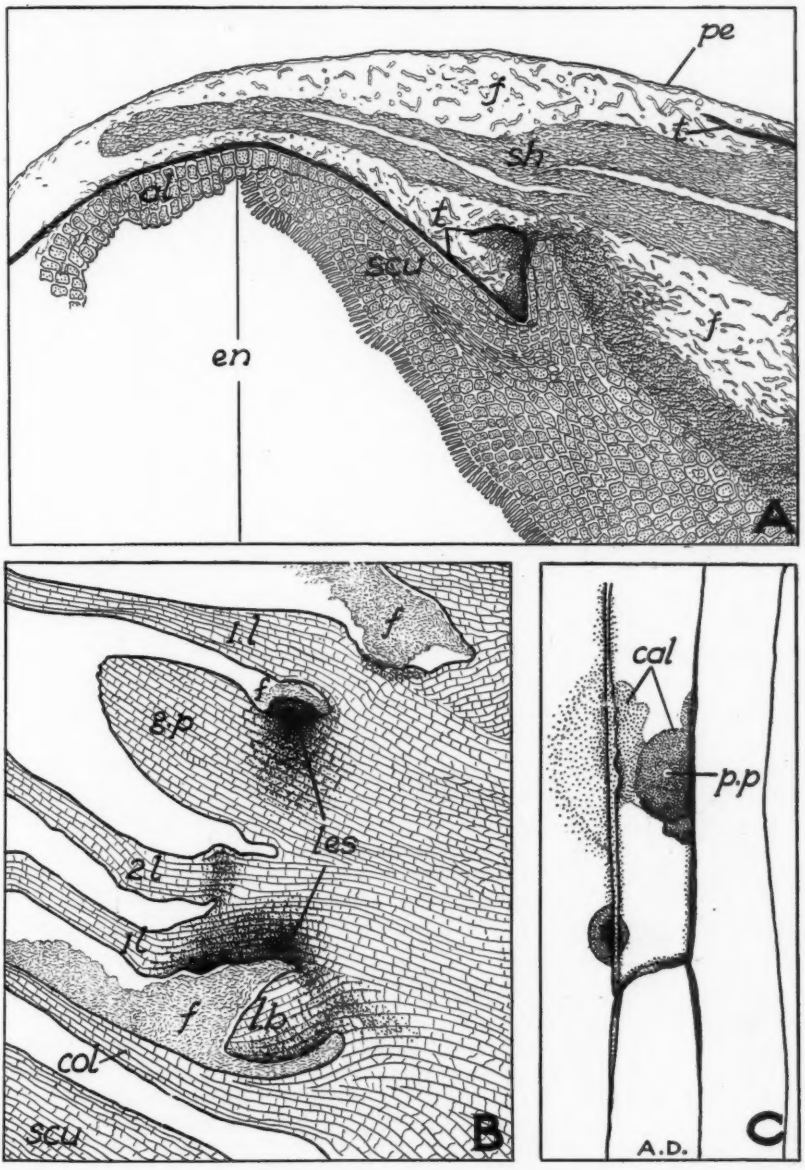


FIG. 13. *Infection of barley seedlings by a seed-borne fungus, H. sativum. A, a longitudinal section of the upper portion of a blighted plumule (sh), showing the testa (t) ruptured, permitting entry of the fungus (f) from the pericarp (pe). The aleurone (al), scutellum (scu) and endosperm (en) were not infected, ×170 approx.; B, a longitudinal section of the region of the growing point, showing lesions (les) at the base of the growing point (g.p) first leaf (1 l) and lateral bud (l.b). The fungus (f) is massed in the axils of these organs, ×185 approx.; C, an infected coleoptile cell showing callosities (cal) and thickening of the cell walls in the region of the penetration point (p.p) and on a side wall, ×1700 approx.*

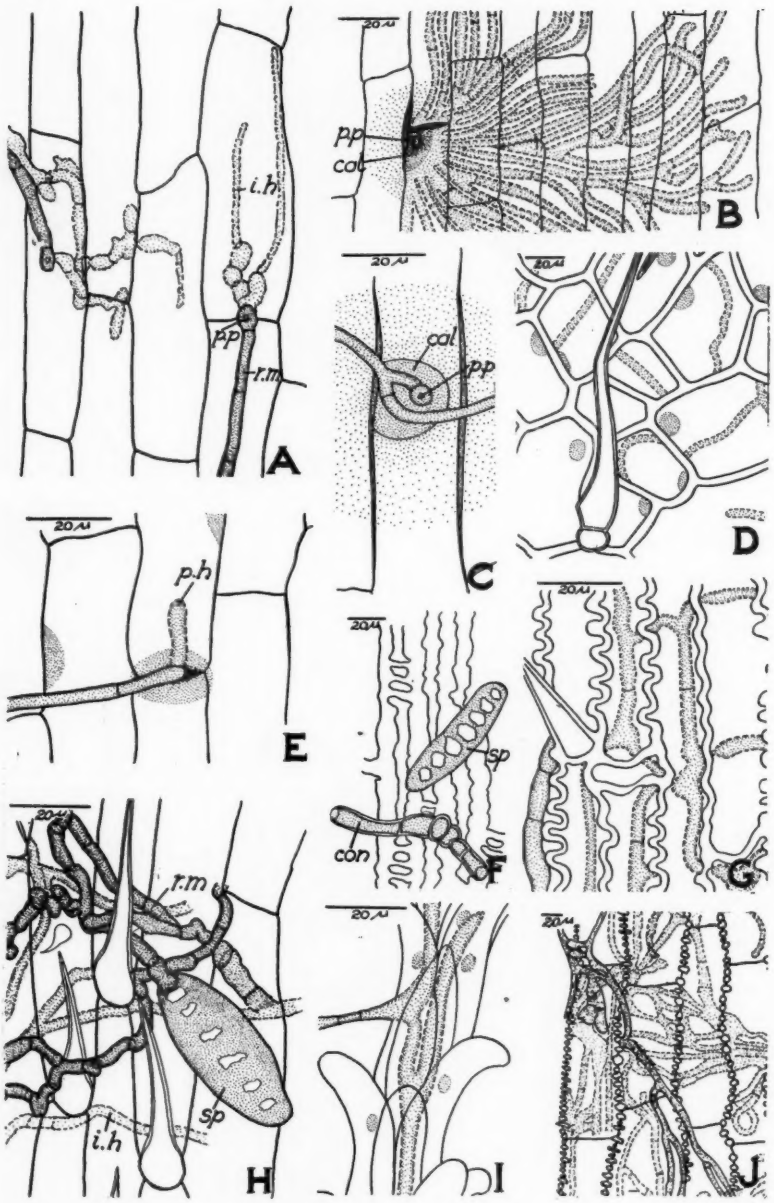


FIG. 14. *H. sativum in tissues of barley kernels. A, resting mycelium (r.m) on the surface of pericarp tissue, producing infection hyphae (i.h) within the cells, 24 hr. after diseased kernels were moistened; B, mycelium spreading in the pericarp from a penetration point (p.p); a callosity (cal) surrounds the penetration point; C, infection of a cell of the coleoptile during germination; D, mycelium in cells of the lodicules; E, penetration of a pericarp cell at a cross wall, showing the penetration hypha (p.h) in the cell; F, conidiophore (con) of H. sativum growing from an opening in the outer epidermis of the lemma; a spore (sp) lying nearby; G, mycelium in the inner epidermis of the lemma; H, a spore (sp) of H. sativum producing mycelium on the surface of pericarp tissue and within the cells; I, mycelium in the stigma; J, dormant mycelium on the surface and within the outer epidermal cells of the pericarp.*

and more diffuse toward the margin, represented what Dufrenoy (6) called a sheath which envelops the penetration point. Others (20, 22) called these structures calluses or callosities (Fig. 13C, *cal*). Invasion of the cells did not appear to cause their death immediately. Several cells were observed which contained hyphae but looked like healthy cells in other respects. For the most part, however, the invaded host cells had collapsed and contained a brown deposit. There was some indication of cell reaction in advance of the hyphae also. This was in the form of a slight yellowing of the cell walls and contents.

Mycelium was found in the remnants of the lodicules also (Fig. 14D). These structures, according to Peyronel (14) contain abundant nutrient materials at flowering time and might serve as a favourable substratum for a fungus. He considered that the rapidity of desiccation of the lodicules would affect the amount of disease in barley seed in different seasons.

3. Occurrence of Spores between the Glumes

Spores of *H. sativum* were found between the lemma and palea where the glumes overlap. In many instances, these spores had germinated and produced mycelium which grew inwards over the pericarp and outwards over the palea (Fig. 14H, *sp*).

C. Examination of Artificially Inoculated Kernels

Two methods of inoculation were used. In one, heads of barley grown in the greenhouse were dusted with finely ground oat hull inoculum of *H. sativum*. Some of these were incubated immediately in a humid atmosphere, others were left dry for various periods before incubating them. The other method involved spraying heads with a spore suspension and then incubating them in a moist chamber. The tissues were examined by taking strips, and making hand and microtome sections.

The following observations were made:

1. Heads Inoculated during Flowering or One to Two Days Later

Heads were inoculated, incubated for 48 hr., and then were examined. The fungus grew profusely over the surface of the glumes and sporulated. Strands of mycelium grew under the edge of the lemma and sporulated in the space between the lemma and palea. Penetration of the glumes was observed. The branches of the feathery stigma were infected early but the cells remained alive for a few days (Fig. 14I). The ovaries soon collapsed and became brown. Mycelium was observed in the embryo sac (Fig. 10, *f*) and in the lodicules (Fig. 14D). When dry inoculum was used, and the heads were left dry for seven days and then were incubated, it was found that fertilization had taken place and that the ovary had developed more or less normally but bore numerous lesions. The cells of the outer epidermis of the pericarp in the lesioned areas were packed with mycelium (Fig. 14J) the strands of which appeared to mass along the cell walls and break through as separate hyphae. These strands were irregular in diameter, and septa were distinguished with

difficulty. Hyphae were found among the cells of the inner layer of the pericarp. These hyphae also were very irregular in outline. Mycelium was seen in the outer epidermis of the lemma. The hyphae were reduced in diameter as they passed from cell to cell (Fig. 14G). Spikelets inoculated and incubated at flowering time were killed by the fungus.

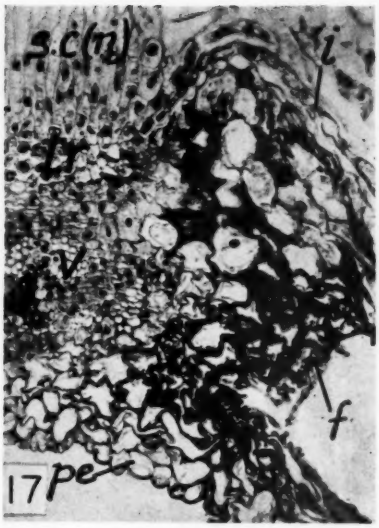
2. Heads Inoculated 7 to 10 Days after Flowering

Mycelium was present throughout the tissues of the glumes and over their surfaces but continuity of the strands was not established. This surface mycelium passed between the edges of the glumes and spread over the pericarp. Points of penetration were observed, and the mycelium spread through the pericarp tissues (Fig. 14B). Freshly invaded cells were seen which had not collapsed, but there were also numerous lesions involving discoloured collapsed tissue. Most of the points of infection were over vertical walls (Fig. 14E) and it was evident, from the staining reaction, that there had been a chemical change in the cell wall for a considerable distance from the point of penetration. Microtome sections showed that the lesions in the pericarp were of considerable depth but that the fungus had not penetrated the inner integument (Figs. 17 and 18). The embryo, endosperm, and aleurone were free of mycelium. The kernels that developed were somewhat shrunken and discoloured.

3. Heads Inoculated 15 to 20 Days after Flowering

At this time, the glumes were adhering to the pericarp and were losing their green colour. The tissues of the glumes appeared to be fairly free of hyphae. There were hyphae in and on the surface of the pericarp, near both ends of the kernels, with very little on the central portions of the ventral and dorsal surfaces. This indicates that the ripening glumes acted as a barrier to the mycelium except at the ends where they are not completely closed. Penetration by the hyphae apparently had not gone beyond the pericarp. No hyphae could be found in the embryo, aleurone, and endosperm. The kernels that developed were plump, but were slightly discoloured.

These studies indicate that if inoculation of hulled barley kernels by a virulent form of *H. sativum* occurs at or soon after flowering, the ovaries are killed, and the other parts of the spikelet become infected. If inoculation occurs from 5 to 10 days later, the glumes, pericarp, and lodicules are invaded and areas of brown dead tissue occur in these organs. These areas contain living mycelium which is thick-walled and highly septate. Penetration of hyphae beyond the inner integument was not observed. Usually there was some shrivelling of the kernel at this stage, possibly as a result of the early collapse of infected pericarp tissues and consequent loss of food materials for the embryo and endosperm. It was observed frequently that filling of heads inoculated at that period was not complete. Some infection of 15- to 20-day-old kernels occurred, but it was confined mostly to the pericarp and to the parenchyma of the glumes at both ends of the kernel. There was no shrivelling at this stage.



FIGS. 15 TO 18. *Infection of barley by H. sativum.* FIG. 15. *Mycelium of H. sativum (f) massed between the coleoptile (col) and the first leaf (1 l) and within the cells of the latter, in a seven day old seedling from naturally infected seed. ×350.* FIG. 16. *Mycelium of H. sativum (f) massed on the surface and within the cells of the coleoptile (col) of a seven day old seedling of barley, from a naturally infected kernel. ×350.* FIGS. 17 AND 18. *Cross sections showing lesions in the ventral and dorsal pericarp (pe), respectively, of spikelets inoculated with H. sativum seven days after flowering. The fungus (f) did not penetrate the chlorophyllous layer (c) of the pericarp or the inner integument (i). Al = aleurone; en = endosperm; ep.n = epidermis of nucellus; s.c(n) = sheaf cells (nucellus); tr = transchalazal tissue; v = vascular trace of the pericarp.* FIG. 17, *×200;* FIG. 18, *×375. Stained with erythrosin and malachite green.*

Studies of Diseased Barley Kernels during Germination

The following observations were made on diseased barley kernels and seedlings grown on moist blotters and in soil.

When the dry mature kernels come in contact with moisture they absorb it through their coverings. The pericarp and testa are thinnest over the embryo, and that is where the most rapid absorption occurs. When sufficient moisture has been absorbed (about 30% of the weight of the kernel in wheat (13)), and if oxygen is available and the temperature favourable, growth of the embryo begins. The first rupture of the testa and pericarp is over the expanding coleorhiza which protrudes and is in turn ruptured by the primary and lateral roots. The coleorhiza in its distended condition consists of thin-walled, elongate parenchymatous cells between which are many intercellular spaces. The coleoptile, through growth, expands towards the apex of the kernel. It ruptures the testa and aleurone at the upper end of the scutellum and forces its way longitudinally within the tissues of the pericarp (Fig. 1, D, E, F; Fig. 13A).

The same conditions that cause the kernel to germinate, cause the dormant mycelium and spores carried in the various tissues to start growing. It can be recalled that the tissues and organs that are in close contact with the growing embryo, viz. pericarp, glumes, and lodicules, were found to contain mycelium. The development of this mycelium in germinating kernels was studied by examining the surface of the kernels, strips of the pericarp, coleorhiza, roots, and coleoptile. The withered lodicules and the base of the shoot were examined also. Strips of the outer epidermis of the pericarp over the dorsal surface of kernels that have been incubated on moist filter paper in Petri dishes for 24 hr. contained both thick-walled resting mycelium and thin-walled new hyphae (Fig. 14A, *r.m*, *i.h*). The thick-walled mycelium was on the surface of the tissue and swollen ends or short side branches were seen overlying penetration points. From these points, new thin-walled hyphae were seen inside the cells. They grew mostly lengthwise of the cells, but they were crossing them also, in their passage to neighbouring cells. In some cases, there was massing or swelling of the hyphae before they broke through the cell walls. The invaded cells were not killed immediately, but later they became necrotic, with brown contents and walls. When pericarp tissue of kernels, germinated at 24° C. for five days, was examined, it was found to contain hyphae throughout its thickness. The coleoptile enclosing leaf primordia and the growing point, forces its way through such infected tissue (Fig. 1, D, E, F) and is exposed to living hyphae during the passage (Fig. 13A, *f*). The duration of this exposure depends upon the soil environment. Similarly, the coleorhiza and roots are in contact with the frayed edges of the pericarp which they rupture. Observations made on this torn pericarp showed hyphae growing from its edges and in contact with the coleorhiza where penetration points were found. Other hyphae grew from the forward edge of the coleorhiza across a considerable space to the surface

of the primary root where they ended in short branches. Strips from the surface of the root contained penetration points. Similarly, hyphae grew from beneath the lemma at the distal end of the kernel, and spread over the coleoptile, where penetration occurred (Fig. 14C, *p.p*). Other internal sources of inoculum are the lodicules. Located as they are at the base of the kernel, they serve as a point of attack upon the coleorhiza and roots. In germinating kernels these bodies were found very frequently covered with conidiophores and conidia of *H. sativum*. The roots and shoot may be attacked from the surface inoculum also. As already described, the fungus sporulates freely on the surface of diseased kernels (Fig. 11). Some of this mycelium comes from beneath the edge of the lemma, and some through the lemma (Fig. 14F, *con*). Direct examination of this surface growth showed that strands of the mycelium were stretched across the space between the ends of the glumes and the surface of the coleoptile, coleorhiza, or roots. Detached conidia were found floating in films of water which had formed along the kernel and coleoptile, and penetration points on the coleoptile were found at this location.

A careful examination of the hypocotyl region of the embryo (Fig. 1, D, E, F, *hy*) of germinating kernels was made. In some cases, dark lesions of considerable depth were present on the dorsal surface of this region (Fig. 19, A, B), and when portions of the discoloured tissue were crushed and examined, mycelium was found in the external layers. Considerable discoloration was seen on the dorsal surface of the distal portion of the scutellum, where it is in contact with the base of the coleoptile. On the surface of this depression strands of dark heavy-walled mycelium were found. There was a tendency for the hyphae to form in clumps (Fig. 16, *f*). Longitudinal sections showed that the tissues of the coleoptile adjacent to these clumps had been invaded to a considerable depth. The mycelium was seen to be massed between the coleoptile and the first leaf; infection of the latter had taken place (Fig. 15, *f*). Similarly, mycelium was seen in the axils of the coleoptile, first and second leaf, and growing point (Fig. 13B, *f*). This mycelium had invaded the tissues at these points and areas of collapsed tissue were evident. The fungus had been able to reach these points when the testa was broken by the expanding shoot (Fig. 13A, *t*, *sh*). This was the condition in the seedlings in which growth of the plumule stopped soon after it had started, but whose roots usually were fairly healthy. More extreme cases were found, in which shoot and roots were dead and filled with mycelium. Usually, the fungus was sporulating on the surface of the dead tissues. As has been stated, most of the seedlings emerged from the soil and continued to grow. Of these, a few produced one leaf and then died. An examination of these showed that the base of the coleoptile, and the leaves and growing point were severely lesioned, decayed, and full of mycelium. In almost all seedlings of this type, the root system was sound at first but soon died as the result of infection and starvation. The remainder of the seedlings developed more or less normally.

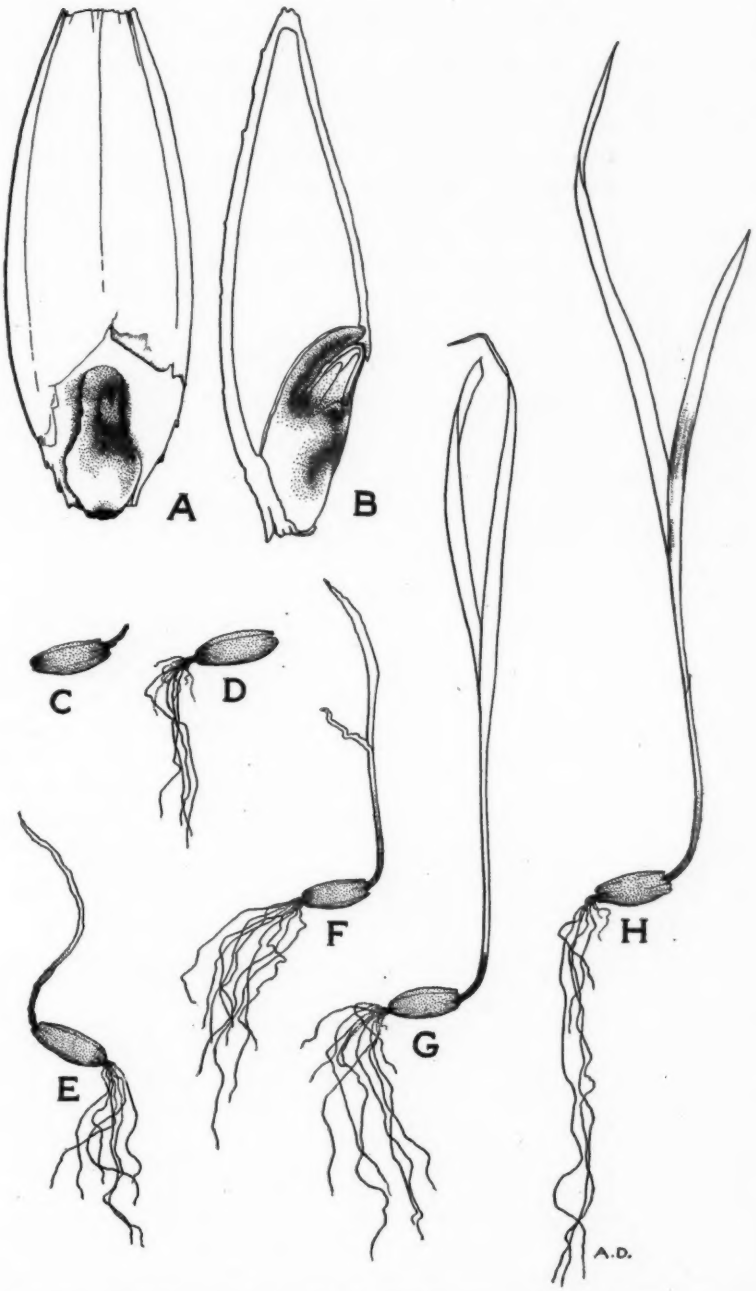


FIG. 19. *Types of seedling injury by a seed-borne fungus, H. sativum. A, B, dorsal view and median section of embryo killed by the fungus, ×9 approx.; C, D, E, pre-emergence blighting; F, postemergence blighting; G, severe basal lesioning and stunting; H, slight basal and foliar lesioning, ×1 approx.*

Their tissues were free of fungi entirely, or they were infected superficially and had surface lesions on the leaves, crown, subcrown internode[1], and roots.

The types of infected seedlings mentioned above are considered to represent five degrees of infection that may occur during germination when the seed is carrying *H. sativum*. These are as follows: (1) complete blighting of plumule and radicle at a very early stage (Fig. 19, A, B, C, D); (2) early blighting of the unemerged shoot with infection soon spreading to the roots (Fig. 19E); (3) postemergence blighting of shoot and roots (Fig. 19F); (4) stunting due to basal infection (Fig. 19G); (5) surface lesioning of shoot and roots with fairly strong growth (Fig. 19H).

Spread of the Fungus from the Seed into the Soil

When the diseased kernels were put in a moist chamber or on the surface of potato dextrose agar in a Petri dish at 24° C., hyphae of the fungus appeared within 24 hr., grew rapidly, and projected above the substratum into the moist atmosphere. It was impossible to demonstrate a similar growth in soil, except in an indirect manner. For this purpose, surface sterilized kernels of barley infected with *H. sativum*, and Ceresan treated[2] kernels to serve as healthy controls, were planted in moist sterile soil in Petri dishes. After four days the germinated kernels were uncovered and particles of the soil lining the pockets in which the kernels lay, were transferred aseptically to potato dextrose agar. At the same time particles of the same lot of sterilized soil were plated. The soil surrounding the diseased seeds produced colonies of *H. sativum*, whereas the check lots of soil particles did not do so. The inoculum that produced the colonies of *H. sativum* on agar could have been hyphae that penetrated the soil, or spores from the surface of the kernels. In order to demonstrate the presence of hyphae in the soil, surface sterilized diseased kernels were placed in contact with the ends of clean glass slides (4) in sterilized soil and the slides were examined four days later. Hyphae of a fungus had adhered to the glass and had grown for distances as great as 2.5 cm. from the end of the glass. These hyphae bore a few conidia of *H. sativum* on short branches.

The Effect of Seed Infection on Later Development of the Barley Plants

Experiment I

These studies were conducted in the greenhouse and in field plots. In the first experiment in the greenhouse, wooden forms with a removable wall were used, filled with a mixture of loam soil and sand. Untreated kernels of barley, infected with *H. sativum*, were planted 2 in. deep and the soil was kept moist. At intervals of one week, the side of the form was removed, and with a gentle stream of water, the soil was washed from the shoot and the upper half-inch of the roots, and the condition of these parts recorded. Then the side of the form was put in position and some of the same mixture of soil was placed around the plants and moistened with water. A summary of the data obtained is given in Table II.

[1] *The internode above the point of attachment of the coleoptile.*
[2] *Ethyl mercury phosphate.*

TABLE II

A COMPARISON OF THE DEVELOPMENT OF THREE GROUPS OF BARLEY SEEDLINGS FROM 38 SEEDS WHICH WERE INFECTED WITH *H. sativum*

Group	Age, wk.	Height, cm.	Seminal roots, No.	Crown roots, No.	Leaves, No.	Tillers, No.	Vigour	Lesioning		
								Coleoptile[1] or subcrown internode[2]	Roots	Leaves
A (25 plants)	1	14.5	5.7	0	2	0	All strong	23 sl.	4[3]	0
	2	20.7	6.0	0	3.7	1	24 strong, 1 weak	15 sl., 1 sv.,	9	8 sl.
	3	32.7	6.0	4	4	1.3	24 strong, 1 weak	14 sl., 2 m., 7 sv.	15	15 sl.
	4	43.0	6.0	8	7	2.1	24 strong, 1 weak	9 sl., 8 m., 7 sv.	18	First leaf dying on all plants
B (3 plants)	1	7.0	5.0	0	2	0	All stunted	1 sl., 2 sv.	1	2 sl.
	2	14.5	5.0	0	2	0	1 weak, 2 dying	1 m., 2 sv.	3	3 sv. on 1st leaf
	3	14.5	5.0	0	3	0	1 weak, 2 dead	3 sv.	3	1st and 2nd leaf dying
	4	14.5	5.0	0	3	0	3 dead	3 sv.	3	Dead
C (10 plants)	1	3.1	6.0	0	Non-emerged from coleoptile	0	All weak, distorted	2 sl., 8 sv.	1	Non-emerged from coleoptile
	2	3.1	6.0	0	0	0	All dead			

NOTE: sl. = *slight;* m. = *moderate;* sv. = *severe.*

[1] *Coleoptile first week.*

[2] *Subcrown internode second, third, fourth weeks.*

[3] *Number of plants with lesions on roots.*

The results indicate that most of the plants (Group *A*) escaped severe primary infection and continued to grow strongly, although what infection there was spread gradually over the base of the plants and was becoming severe at the fourth week. This took the form of lesioning and rotting of the coleoptile, the cortex of the primary roots and subcrown internode, and blighting of the first leaf. Development of leaves and crown roots continued and the latter remained free of lesions. Generally, the growth of these plants appeared to be affected only very slightly by the fungous infection when the final data were taken. Group *B* included plants that were infected early but were able to emerge from the soil. Shoot and root development gradually stopped and the plants were dead by the fourth week. In Group *C*, 10 plants failed to emerge and died very quickly. It was observed, however, that the primary roots of these plants were quite healthy until the shoot died. Thus, under greenhouse conditions, 13 out of 38 plants from seed infected with *H. sativum* failed to survive beyond the fourth week, the majority of them having failed to emerge. The remainder, 25 plants, were growing strongly when the experiment was terminated, but many of them bore extensive basal lesions. The plants that died early showed signs of serious injury before the end of the first week whereas those that continued to grow were either free of infection or were infected lightly, at that time. It was observed, however,

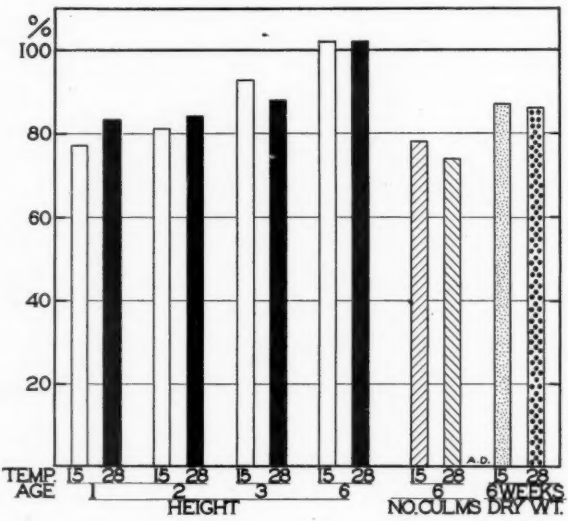


FIG. 20. *Bar diagram comparing the development, under controlled conditions, of barley seedlings from healthy seed and from seed naturally infected with H. sativum. The data for the diseased seedlings are represented as percentages of equivalent data for the healthy seedlings which are shown by a horizontal straight line at 100%.*

that in all cases where there were basal lesions on the coleoptile during the early stages of growth, the subcrown internode and roots became lesioned later. This infection spread rapidly on some plants. The further growth of the plants was related fairly closely to the extent of infection and the rapidity of its spread.

Experiment II

In another experiment conducted in the greenhouse, seed of the same diseased sample of barley was sown in soil in 1 gal. earthenware crocks that were suspended in water-baths. Untreated kernels were planted in one-half of the containers, and kernels treated with Ceresan were planted in the others. The soil moisture was maintained at 60% of its moisture holding capacity by regular weighings, and temperatures of 15 and 28° C. were maintained in water-baths. Ten kernels were sown 1½ in. deep in each container. The height of the plants was measured at one, two, and three weeks after sowing, and again at the sixth week, when the number of culms and dry weight also were recorded. The data for the plants from untreated seed are represented graphically in Fig. 20, as percentages of the corresponding data for plants from treated seed, which are rated at 100%.

Under controlled greenhouse conditions where the plants from untreated seed did not have to compete with plants from treated seed, there was a gradual recovery in height by the diseased plants, following an initial set-back. However, the plants from untreated seed had fewer culms and were lighter in weight than those from treated seed. Sallans (19) found that under favourable conditions wheat seedlings showed definite signs of recovery from an initial severe set-back by *H. sativum*.

Experiment III

An experiment was conducted in the field with seed from the same sample sown in rows six inches apart. A plot consisted of one row of untreated seed, and one row of treated seed, and it was separated from the next plot by a row of treated seed. There were 16 plots arranged in four by four Latin square. This provided sufficient material for four dates of harvesting, each having four replicates. A count of the emerged seedlings was made 14 days after seeding. The mean emergence in the 16 untreated rows was 39% and that in the 16 treated rows 94%. At this time all of the plants in four replicates were pulled to be used for measurement of height and dry weight, and for making counts of culms and observing disease symptoms.

The plants in the treated rows of the remaining plots were thinned so as to leave the same number as had emerged in the untreated rows. The rows separating the plots were not thinned and their heavy growth provided strong competition for the thinned rows. Quadruplicate plots were harvested at

28, 42, and 80 days. The data from the untreated rows are represented graphically in Fig. 21, as percentages of the corresponding data from the treated rows, which are rated at 100%.

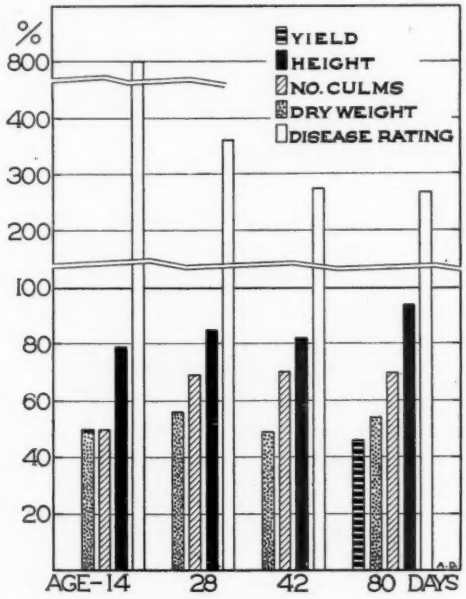


FIG. 21. *Bar diagram comparing the development, under field conditions, of healthy barley seedlings and seedlings from seed infected with H. sativum. The data for the diseased seedlings are represented as percentages of equivalent data for the healthy seedlings, which are shown by a horizontal straight line at 100%.*

Under field conditions, where there was strong competition for moisture with neighbouring unthinned rows of healthy plants, the plants from untreated seed were weaker throughout the season than those from treated seed. The 14-day old diseased plants had a high disease rating[1] and were stunted and light in weight. As time passed they compared more favourably with the healthy plants. The latter became lesioned as the season progressed, but maintained their superiority in height, number of culms, and weight. Their yield was more than twice as great as the yield from the plants in the untreated rows. These results indicate that under field conditions where there was strong competition for moisture, the effects of an early severe attack by *H. sativum* on barley seedlings persisted, and were only partially overcome by the plants.

[1] *Rated as follows: blighted, 10; severely lesioned, 4; moderately lesioned, 2; slightly lesioned, 1.*

$$\textit{Disease rating} = \frac{\textit{sum of ratings}}{10} \times \frac{100}{\textit{number of plants}}.$$

The results given above show that the fungus, *H. sativum*, when borne on the seeds, was capable of causing severe injury to the barley seedlings under a fairly wide range of temperature conditions, in the presence of sufficient moisture to give good growth. The most obvious and serious injury was seedling blight. However, light infection also occurred and spread over the base of the plant, involving the roots, subcrown internode, and leaves. The reaction of the individual seedlings to infection varied, but in general, a severe early infection caused stunting and early death. In most cases there was considerable recovery from the effects of infection. This occurred where competition for moisture was not severe. Even when the extent of seed infection was known it was impossible to predict the amount of injury which would be caused by the fungus. This seemed to be a complex result of various factors affecting host and parasite.

Discussion

The relationship between the barley kernel and a fungus parasite borne in and upon the seed coverings is much more intimate than the relation between the kernel and the same parasite in soil. Proximity in itself should make a difference, but there are physiological, nutritional, and probably antibiotic factors also which are quite different. A fungus within the seed coat has certain food substances immediately available on germination of the seed, which are not available to soil fungi. In its relation to other microorganisms, the seed-borne fungus of barley is protected to a greater extent than the soil-borne fungus. Competition for food is not so acute, and antibiosis probably is less effective because of the hard seed coverings.

Barley kernels may become infected with *H. sativum* during maturation. The commonest forms of inoculum are air-borne spores and fragments of mycelium. These may come from neighbouring crops, grasses, or crop refuse. The time of inoculation is important, because the survival and further development of the kernels depends upon it. It has been shown that spikelets inoculated at flowering time usually are killed outright, while later inoculation may cause only shrivelling and discoloration. The opportunities for infection by air-borne conidia become limited by the changes in the tissues during development. The floral glumes become hard in texture and after the third week following flowering adhere to the pericarp. This is an important barrier to the entry of inoculum. In addition to this, the caryopsis steadily develops a resistant membrane, the testa, which becomes more resistant as time goes on. The spread of the fungus is likewise limited by the natural collapse of the tissues, with a consequent draining of organic nutrient materials and moisture which might be used by the fungus.

The fungus does not live in complete compatibility with the tissues of the barley kernel. Some host cells attempt to prevent penetration by formation of callosities. Those that become colonized eventually die and collapse. The mycelium in the dead cells remains viable and germinates to produce hyphae that infect other cells, or forms conidiophores and conidia. The cells

that are killed, singly or in groups, become discoloured and constitute lesions. Strands of the fungus may, however, remain on the surface of cells or in intercellular spaces without any apparent harm to the cells. The surface mycelium usually is thick-walled and its protoplasm may become vacuolated.

The distribution of the fungus in the tissues bears a direct relation to infection of the seedling. The plumule and radicle of the embryo rupture the testa and pericarp of the kernel and remain in contact with diseased tissue. This includes pericarp, lodicules, and glumes. Rupture of the testa permits spread of the fungus from the pericarp to the base of the plumule and radicle. This may be followed by rapid invasion of the growing point, lateral bud, and primary roots. Usually, however, the fungus requires considerable time to penetrate the coleoptile and primary leaves in order to reach the growing point. Similarly, the coleorhiza blocks the progress of the fungus towards the roots for a short time. Thus, the speed of penetration of the outer sheaths of the embryo determines to some extent the fate of the shoot and roots. These may be killed or may continue to grow and bear lesions in their cortical tissue. These lesions provide inoculum for secondary infection.

A variable amount of stunting of infected seedlings was evident from the beginning of their development. This was caused by destruction of certain tissues and probably by toxic action of the fungus and competition for food. Recovery took place gradually if nutrient materials and moisture were available but many of the infected plants suffered permanent injury in the form of reduced root growth and tillering. The results of a field experiment under dry conditions were indicative of the importance of competition for moisture. In that experiment, unthinned rows of healthy barley flanked the thinned rows of diseased and healthy barley. The result was that the diseased plants were much inferior to corresponding healthy plants throughout the season.

Acknowledgments

The author is indebted to Dr. P. M. Simmonds, Dr. D. L. Bailey, and Dr. H. B. Sifton for guidance during the course of these investigations. The assistance of Mr. A. W. Davey in preparation of some of the figures is gratefully acknowledged.

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THE HAEMOLYTIC ACTION OF PHENOTHIAZINE DERIVATIVES[1]

BY H. BRUCE COLLIER[2] AND DELLA E. ALLEN[3]

Abstract

The haemolysis of horse erythrocytes by saponin or by lysolecithin, *in vitro*, is powerfully accelerated in the presence of phenothiazone, thiazine S-methyl sulphonium perchlorate, and especially the urinary conjugate, potassium leucophenothiazone sulphate. Bile salt haemolysis is only slightly affected.

Sheep cells are more resistant to haemolysis and to its acceleration by these derivatives.

Phenothiazone does not produce photodynamic haemolysis.

This acceleration of haemolysis is suggested as an explanation of the haemolytic anaemia frequently observed in some species after treatment with phenothiazine.

Introduction

Phenothiazine, when used as an anthelminthic, is relatively non-toxic in sheep (25) and most other laboratory and domestic animals (28). In horses, however, relatively small doses of the drug may cause an acute haemolytic anaemia; the nature of this anaemia has been studied by Swales, Collier, and Allen (27). In humans, also, a haemolytic anaemia frequently results from phenothiazine therapy (13, 17) and one fatality has been recorded (1).

The recent review of Haden (12) indicates that little is known of the mechanism of the anaemias caused by drugs. In the case of the phenothiazine derivatives, tests in this laboratory have failed to reveal any direct haemolytic action upon horse erythrocytes *in vitro*. Finally, however, advantage was taken of the technique of Ponder (19) to demonstrate that some of the soluble derivatives *accelerate* the haemolysis of red cells that is produced by lysins such as saponin and lysolecithin. The hypothesis is advanced that the drug may exert its haemolytic action *in vivo* through this accelerating effect.

Experimental

Materials

The saponin used was the product of Eimer and Amend. Lysolecithin was prepared from egg yolk by the method of King and Dolan (15), using rattlesnake venom* as the source of lecithinase.

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Contribution from the Institute of Parasitology, McGill University, Macdonald College, Que., with financial assistance from the National Research Council of Canada.

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* *We are indebted to the Technical Division, Sharp and Dohme, Glenalden, Pa., for a generous supply of dried Crotalus atrox venom.*

Phenothiazone (3-oxyphenothiazine) was prepared by the method of Pummerer and Gassner (21). N-methyl phenothiazine and thiazine S-methyl sulphonium perchlorate were made according to Bernthsen (3) and Kehrmann and Dardel (14) respectively. Conjugate—potassium leucophenothiazone sulphate—was recovered from the urine of treated sheep and horses by the method of Collier (7) and was recrystallized several times from water. Synthetic conjugate was prepared from leucophenothiazone by the method of Burkhardt and Lapworth (6).

Methods

Horse and sheep blood were collected directly into a preservative anti-coagulant fluid and were stored at about 4° C. The fluid first used was the glucose–citrate solution of Denstedt (11), but washed horse cells were found to be somewhat unstable when suspended in it. Finally, Denstedt's (10) modified preservative fluid* was found to be quite suitable. This solution was used throughout for storing, washing, and finally suspending the cells.

One volume of blood was collected into ½ volume of this fluid. The cells, before testing, were washed three times and were made up to a concentration of 1 : 3 by volume. To 10 ml. of the solution was added a suitable small volume of stock lysin solution (saponin or lysolecithin) and 0.10 ml. of the cell suspension, giving a final concentration of red cells of 1 : 300 by volume. In testing a compound for acceleration, it was first dissolved in the fluid, then the lysin and cells were added, in that order.

Haemolysis was allowed to proceed at room temperature, the amount of lysin being sufficient to cause complete haemolysis in about 30 to 60 min. The breakdown of the cells was followed by observing the change in opacity in a Klett-Summerson photoelectric colorimeter equipped with a red filter (Corning No. 241). The reading at 100% haemolysis gave a blank value which was used to calculate percentage haemolysis from the other readings. As seen in Fig. 1 the haemolysis curves are sigmoid; 50% haemolysis was taken as the end-point, because of the difficulty in estimating the exact time for complete lysis.

The time for 50% haemolysis of a standard cell suspension by a known amount of lysin was first determined. Then the time required for the same amount of lysin to cause 50% haemolysis in the presence of the test substance was ascertained. (In no case did the test substance alone, in the absence of lysin, cause haemolysis.) The acceleration was then expressed as the quotient, Q = time with lysin alone ÷ time with lysin plus accelerator.†

* *100 ml. 5% glucose.*
75 ml. 3% sodium citrate.
75 ml. isotonic phosphate buffer, pH 7.4.

† *This factor is roughly the reciprocal of Ponder's "R," which is the ratio of lysin concentrations necessary to give complete haemolysis in the same period of time, i.e., concentration in presence of accelerator ÷ concentration in absence of accelerator.*

As the main purpose of the present paper is to point out the accelerating effect of the phenothiazine derivatives, it was found simpler to employ a fixed lysin concentration and to observe the difference in time interval. It is planned to investigate the kinetics of this acceleration in greater detail, at which time the conventional methods of expression will be employed.

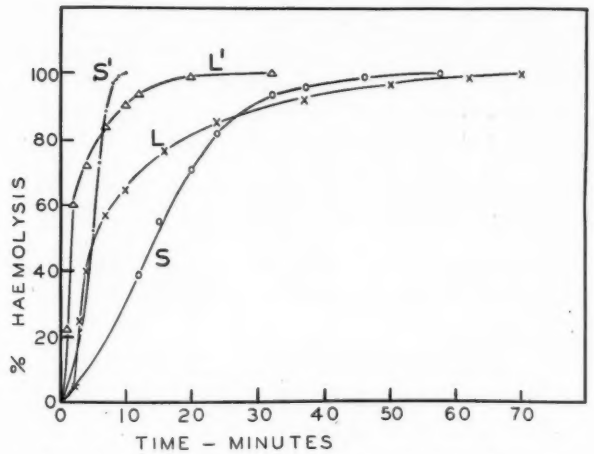

FIG. 1. *Haemolysis of horse erythrocytes. S, saponin alone; S', saponin + 2 × 10⁻⁵ M phenothiazone; L, lysolecithin alone; L', lysolecithin + 3 × 10⁻⁵ M phenothiazone.*

Acceleration of Saponin Haemolysis of Horse Cells

Fig. 1 illustrates the nature of the time–haemolysis curve obtained by the action of saponin on horse erythrocytes. It demonstrates also the accelerating effect of phenothiazone at 2×10^{-5} *M* concentration. Table I gives the relationship between the concentration of phenothiazone and the degree of acceleration observed.

TABLE I

EFFECT OF PHENOTHIAZONE ON SAPONIN HAEMOLYSIS OF HORSE CELLS

Cells, 1 : 300; saponin, 25 μg. per ml.

Phenothiazone concentration, $M \times 10^6$	Time for 50% lysis, min.	Q
0	14	1.0
2.0	13	1.1
7.4	9	1.6
20	5	2.8
35	3	4.6
74	2	7.0

Cells suspended for 30 min. in 3×10^{-5} *M* phenothiazone and then washed were no more fragile to saponin than were untreated cells. (It was noted that some methaemoglobin was formed, indicating penetration of the cells.) Ponder (18) similarly found that cells treated with solutions of benzene and of indole showed no permanent effects if washed immediately.

The conjugate isolated from horse urine was found to exert a very powerful accelerating effect; the same was true of the synthetic conjugate. As it is

impossible to obtain these derivatives in a high degree of purity, the results for the two were not identical, as seen in Table II. Furthermore, the concentration of conjugate was found to be very critical, which made it more difficult to obtain precise Q values.

TABLE II

EFFECT OF CONJUGATE ON SAPONIN HAEMOLYSIS OF HORSE CELLS

Accelerator	Time for 50% lysis, min.	Q
Horse conjugate,		
0	30	1
1.1×10^{-5} M	10	3
5.6×10^{-5} M	< 1	> 30
Synthetic conjugate,		
0	25	1.0
3×10^{-5} M	30	0.8
3×10^{-4} M	11	2.3
3×10^{-3} M	1	25

Other compounds, listed in Table III, were tested, but in this series the effect of varying concentration was not determined.

TABLE III

ACCELERATION OF SAPONIN HAEMOLYSIS OF HORSE CELLS

Substance	Concentration, M	Q
Thiazine S-methyl sulphonium perchlorate	0.0016	10.9
N-methyl phenothiazine	0.0012	1.2
Quinine	0.0005	6.8
Indole	0.0020	2.7
Sulphanilamide	0.010	1.5
Acetylcholine	0.0020	1.0

Haemolysis of Sheep Cells

Sheep erythrocytes are generally believed to be less fragile than horse erythrocytes. With saponin, it was found that sheep cells required about three times the concentration to produce the same rate of lysis as horse cells. Further, the accelerating effect of the phenothiazine derivatives was appreciably less, as seen in Table IV.

Inhibition of Haemolysis by Serum

Blum (4) and Tsai and Lee (29) have demonstrated that there is an antihaemolytic substance in normal serum. Under the conditions herein described it was confirmed that very little serum was required to neutralize the amount

TABLE IV

SAPONIN HAEMOLYSIS OF SHEEP CELLS

Cells, 1 : 300; saponin, 75 μg. per ml.

Accelerator	Time for 50% lysis, min.	Q
None	20	1.0
Phenothiazone, 0.0002 *M*	6	3.3
Sheep conjugate, 0.0003 *M*	12	1.7
Sheep conjugate, 0.003 *M*	4	5.0

of saponin used, and that this antihaemolytic effect was also exerted in the presence of the accelerators. The effect of serum upon saponin lysis of horse cells, in the presence and absence of accelerators, is summarized in Table V.

TABLE V

EFFECT OF SERUM UPON SAPONIN HAEMOLYSIS OF HORSE CELLS

Serum concentration	Time for 50% lysis, min.	
	Saponin alone	Saponin plus *M*/1000 conjugate
0	15	Instantaneous
1 : 250	13	Instantaneous
1 : 100	20	6
1 : 80	> 240	> 240

Lysolecithin Haemolysis of Horse Cells

Since lysolecithin is believed to be a normal lysin in the mammalian circulation, the effect of accelerators on its action was examined. This lysin was not investigated extensively, as it was found difficult to obtain reproducible results. This was shown to be due to the fact that the activity of the lysolecithin decreased rapidly in very dilute solution. (This is presumably the reason why the rate of haemolysis falls off so markedly as complete lysis is approached.) Nevertheless, the results obtained were sufficient to demonstrate that the same typical acceleration of haemolysis occurred in the presence of phenothiazone and of the conjugate.

Fig. 1 illustrates the normal time curve of haemolysis of horse cells by lysolecithin (7 μg. per ml.) and the accelerated curve in the presence of 3×10^{-5} *M* phenothiazone; from the times for 50% lysis, Q is found to be about three. The conjugate had a very powerful effect upon lysolecithin action, Q values of approximately 25 to 50 being obtained. Because of the difficulty in obtaining precise quantitative data, the observations are summarized qualitatively as follows:—

Horse conjugate, 1.3×10^{-5} *M*...no acceleration.
1.3×10^{-4} *M*...instantaneous lysis.
Synthetic conjugate, 4×10^{-6} *M*...slight acceleration.
4×10^{-5} *M*...instantaneous lysis.

Serum inhibited lysolecithin haemolysis, and its acceleration, just as in the case of saponin. Serum at 1 : 50 dilution completely prevented haemolysis by the lysin alone; it inhibited also the haemolysis in the presence of lysin and 0.001 *M* conjugate. Serum at 1 : 500 slightly inhibited both normal lysis and that taking place in the presence of conjugate.

Bile Salt Haemolysis of Horse Cells

Horse erythrocytes were lysed in the presence of 0.026% sodium choleate (Merck). Phenothiazone, however, had only a very slight accelerating effect. In the presence of 5×10^{-4} *M* phenothiazone, the time for 50% haemolysis was reduced from 18 min. to 9 min., giving a *Q* value of 2.0.

The Effect of Light

Blum (5) has demonstrated photodynamic haemolysis in the presence of dyes such as rose bengal. It was thought that the phenothiazine derivatives might show a similar behaviour, especially as a photosensitizing action of phenothiazine has been demonstrated by DeEds (9) in humans, and by Swales *et al.* (26) in pigs.

A standard suspension of horse cells in 10^{-4} *M* phenothiazone was irradiated for one hour at room temperature by the light of a small carbon arc, through a water cell, at a distance of 30 cm. There was no detectable haemolysis, compared with a dark control, even 24 hr. after the irradiation. It was, therefore, concluded that phenothiazine derivatives probably do not exert their haemolytic effect through photodynamic action.

Discussion

Administration of phenothiazine to horses may cause an acute haemolytic anaemia, but the various derivatives of the drug that have been tested *in vitro* have no direct lytic effect on horse erythrocytes. It has been shown, however, that some of these compounds have a striking effect in *accelerating* the lysis of the cells by saponin or by lysolecithin, with a much smaller effect upon bile salt haemolysis. This phenomenon is analogous to that described by Ponder (18, 19) in the case of benzene, indole, nonyl alcohol, etc.

It is of interest to note that phenothiazone and thiazine S-methyl sulphonium perchlorate, which are strong accelerators of haemolysis, are also powerful cholinesterase inhibitors (8).

Of especial interest is the very strong accelerating effect of the conjugate, potassium leucophenothiazone sulphate, which is believed to be the derivative of phenothiazine that occurs in the blood of treated sheep (7) and of horses (27). In the horse, we have observed a concentration of conjugate in the plasma of about 0.01%, or 3×10^{-4} *M*, which is sufficient, *in vitro*, to cause

a very marked acceleration of haemolysis. The conjugation of leucophenothiazone to the sulphate is a detoxifying mechanism; nevertheless it may actually intensify the haemolytic effect.

To apply this accelerating effect of the phenothiazine derivatives, observed *in vitro*, to explain intravascular haemolysis involves several assumptions. First, it may be assumed that circulating erythrocytes are normally being broken down by a lysin, such as lysolecithin, as has been proposed by Bergenhem and Fåhraeus (2). (See, however (24).) It would then be assumed that the phenothiazine derivatives in the blood can markedly accelerate this normal cell breakdown.

Support for this hypothesis may be found in the fact that indole is an accelerator of haemolysis (19), and Rhoads (22) has demonstrated that indole is haemolytic in dogs, especially on a vitamin-B–free diet. Furthermore, the accelerating effect of quinine, described by Ponder and Abels (20) and confirmed by us, has been used by Mer and co-workers (16) to explain the acute haemolytic anaemia frequently observed in malaria patients treated with quinine. These workers showed that quinine could accelerate bile salt haemolysis.

The inhibitory effect of serum on erythrocyte lysis may seem to be an objection to this hypothesis. However, conditions in the circulation are not identical with those *in vitro*, and it is believed that the erythrocyte exists in a state of more or less unstable equilibrium, being constantly subject to lytic tendencies. If so, the presence of accelerating drugs may quite possibly hasten the process of lysis, which presumably is taking place continually in the normal animal.

In order to test the effect of the vitamin B complex on the phenothiazine anaemia, rabbits and guinea pigs were kept on a relatively B-free diet. Large and repeated doses of the drug failed, however, to produce any significant degree of anaemia (Swales, Collier, and Allen, unpublished data.) Schnitzer, Siebenmann, and Bett (23) had a similar experience with white mice and guinea pigs on normal diets; with dogs they found no difficulty in producing a marked anaemia.

In conclusion, it seems clear that a more adequate interpretation of the haemolytic anaemias caused by drugs depends upon a more complete understanding of the properties of the normal erythrocyte and of its destruction in the circulation.

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PRESERVATION OF EGGS

II. SURFACE CONTAMINATION ON EGG-SHELL IN RELATION TO SPOILAGE[1]

By F. T. Rosser[2]

Abstract

The method devised to measure surface contamination on shell eggs was to wash eggs with water in a "Waring blendor" and make bacteriological and mould counts on the wash water. To determine the degree of internal contamination, a small electric hand drill was used to cut holes in the shell in order to remove the egg contents under sterile conditions. The egg meats were mixed in the blendor before plating.

External mould growth, after storage for six weeks, under adverse conditions of both temperature and humidity, did not bear any close relation to the amount of mould contamination initially present on the shell. Both oil dipping and handling stimulated the growth of external moulds.

Internal spoilage, as determined by candling at the end of the storage period, was greatest for eggs having a high initial shell count and least for those with a low initial count. Oiling, or disinfecting with hydrogen peroxide followed by an oil dip, did not reduce spoilage. Storage life was increased by strict observance of recognized sanitary practices.

Introduction

It is generally accepted that egg spoilage is usually caused by organisms which enter through the shell (3). If this is true the early elimination of surface contamination followed by sealing the shell pores should considerably reduce losses in storage. This study was undertaken to determine the relation existing between the numbers of bacteria and moulds on the exterior surface of shell eggs and subsequent spoilage in storage, and to assess the value of oil as a sealing agent against the entrance of micro-organisms into the egg.

Methods

Determinations of the number of moulds and bacteria on outer shell surfaces were made by washing eggs in a "Waring blendor"* and plating the wash water on a suitable medium. An open wire basket was constructed to fit closely to the sides of the blendor container and of such a length that the bottom was suspended just above the propeller blades. Three eggs, placed in the basket, were covered by a measured amount of sterile water and the machine operated at high speed for three minutes. Preliminary investigation had shown that further washing removed a negligible number of organisms and tended to raise unduly the temperature of the wash water. Recognized bacteriological technique was used throughout. Eggs were untouched by hand during the experiment, metal lifters being used to move tray lots, and crucible tongs or loops made from stiff wire to manipulate individual eggs. The glass blendor containers and their plastic tops were sterilized in a water-bath the temperature of which was raised to above 80° C. for 10 min. Samples

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* *Manufactured by the Waring Corporation, New York, N.Y.*

of the wash water were plated in duplicate on proteose peptone tryptone agar for bacteria and on potato dextrose agar for moulds. Plates were incubated for seven days at 20° C.

To determine quantitatively internal contamination of eggs it was essential that the contents be removed aseptically. The following procedure was found satisfactory for this purpose. The egg was held in a clamp on a ring stand and both ends were painted with iodine. A circle about one-quarter inch in diameter was cut in the shell at the air cell end with a small electric hand drill and a small hole was bored at the opposite end of the egg, care being taken not to puncture the membranes. The cut surfaces were again treated with iodine, after which the excised portion of the shell and the outer membrane were removed with sterile forceps and the membrane below the small hole at the opposite end of the egg was punctured with a sterile needle. The egg was then inverted over a sterile beaker, the lid of which was removed momentarily, and filtered air under pressure applied to the hole in the small end of the egg. In this way the inner membrane was broken and the contents expelled quickly through the large opening, often without breaking the yolk. The meats from the six eggs in each treatment, except those accidentally cracked, were removed as described, poured into the blendor container and mixed for three minutes. Aliquots (1 cc. and 1/10 cc.) were plated in duplicate for the bacterial count. Methods of examination following storage comprised observation of each egg under a strong light for exterior growths and careful candling before a lamp to detect interior defects. All doubtful eggs were broken open and scrutinized under the light.

Experimental

The storage experiment which was begun during the latter part of February was designed statistically in a 6 × 6 Latin square arrangement of half-case units. Each of the six half-case units contained six trays of eggs, each tray of 30 eggs being representative of one of six treatments. Three dozen eggs were secured for each replication of each treatment, six of which were selected at random for initial determinations and the remainder (30) used for the storage experiment. The treatments were as follows:

A. Market eggs (obtained as required from a local egg grading station)
- (1) Grade A Pullet: untreated.
- (2) Grade A Pullet: given oil dip.
- (3) Grade A Pullet: immersed in 3% commercial hydrogen peroxide, dried, and given oil dip.
- (4) Grade A Pullet: immersed in bath containing average of 75,000,000 bacteria and 600,000 moulds per cc.

B. Producer eggs (from a flock kept under excellent conditions)
- (5) Grade A Medium, day-old: graded and handled by producer in usual manner.
- (6) Ungraded, day-old: collected and candled with dry gloved hands.

A commercial, colourless, odourless, tasteless, paraffin base, egg-dipping oil, having a pour point of 40° F. was used for the oil dipping treatments. Each half-case unit was stored for a total period of six weeks under the conditions, indicated in Table I, which were considered suitable for accelerating egg spoilage.

TABLE I

STORAGE CONDITIONS EMPLOYED FOR HALF-CASE UNITS OF EGGS

Half-case No.	Time stored (days)	
	Alternating every two days: 17.6° C. and 95.7% R.H.* 23.5° C. and 69.6% R.H.	Constant: 21° C. and 90% R.H.
1	32	10
2	29	13
3	28	14
4	27	15
5	26	16
6	25	17

*R.H. = *relative humidity.*

The six eggs selected for initial examination were washed in two batches of three each and the average number of organisms was calculated on a "per egg" basis for each treatment. After the eggs had dried they were stored at 0° C. until it was convenient to examine the contents (in no instance for longer than 11 days).

TABLE II

INITIAL BACTERIA AND MOULD COUNTS ON SHELL EGG SURFACES

Condition before washing	Number of eggs washed	Average count per egg	
		Bacteria	Moulds
A. Market eggs			
Untreated*	72	70,000	1600
Dipped in hydrogen peroxide**	36	730	0
Dipped in contaminant**	36	2,780,000	29,000
B. Producer eggs			
Ordinary handling	36	103,000	43
Handled with gloved hands	36	9500	45

* *Includes eggs for control and oil dip treatments.*
** *Allowed to dry off before washing (one to two hours in laboratory).*

Table II shows the results obtained for exterior contamination on the variously treated shell eggs prior to storage. When the difference in size of the eggs was considered, the bacterial count on Grade A market eggs of unknown history was of the same order as that from producer eggs ordinarily handled, but the mould count was about 30 times as high. The producer

eggs untouched by hand, however, showed approximately one-tenth the bacterial count of those handled whereas the mould counts were almost identical. Following hydrogen peroxide treatment counts for both moulds and bacteria were very low, and after dipping in the contaminated bath, very high.

Examination of egg meats revealed the presence of bacteria in only one group of six market eggs (100 bacteria per cc.). This contamination may, of course, have been contributed by only one of the eggs. No moulds were found in any of the meat samples.

The percentages of eggs having external mould and internal mould or bacterial growths at the end of the storage period are given in Tables III and IV, respectively. Because of the differences in percentage of the various amounts

TABLE III

MOULD GROWTH ON EGG SHELL SURFACES AFTER SIX WEEKS' STORAGE

Treatment	Percentage with mould growth on shell						
	Half-case number						Average
	1	2	3	4	5	6	
A. Market eggs							
Untreated	63.3	40.0	60.0	100.0	60.0	80.0	67.2
Oil dipped	100.0	100.0	100.0	100.0	100.0	100.0	100.0*
Hydrogen peroxide treated and oil dipped	96.7	100.0	100.0	100.0	100.0	100.0	99.4*
Dipped in contaminant	66.7	100.0	100.0	90.0	0.0	70.0	71.1
B. Producer eggs							
Ordinary handling	100.0	100.0	16.7	96.7	100.0	30.0	73.9
Handled with gloved hands	6.7	26.7	36.7	10.0	20.0	90.0	31.7

* *Differed significantly from market eggs, untreated.*

TABLE IV

INTERNAL EGG SPOILAGE AFTER SIX WEEKS' STORAGE

Treatment	Percentage spoilage						
	Half-case number						Average
	1	2	3	4	5	6	
A. Market eggs							
Untreated	10.0	13.3	50.0	63.3	80.0	96.7	52.2
Oil dipped	56.7	40.0	86.7	80.0	86.7	73.3	70.6
Hydrogen peroxide treated and oil dipped	30.0	23.3	43.3	76.7	86.7	93.3	58.9
Dipped in contaminant	40.0	76.7	86.7	86.7	70.0	90.0	75.0*
B. Producer eggs							
Ordinary handling	0.0	6.7	33.3	13.3	83.3	63.3	33.3*
Handled with gloved hands	0.0	13.3	40.0	36.7	30.0	60.0	30.0*
Half-case average	22.8	28.9	56.7	59.4	72.8	79.4	

* *Differed significantly from market untreated.*

TABLE V

ANALYSIS OF VARIANCE OF EGG SPOILAGE

Source of variance	Degrees of freedom	Mean square	
		External mould	Internal spoilage
Half-case replicates	5	194.98	1558.46**
Tray position in case	5	743.77	102.62
Treatments	5	2679.99**	1013.31**
Residual error	20	465.38	117.64

** *Exceeds mean square error, 1% level of significance.*

of spoilage the data were transformed to angular measure according to the formula $p = \sin^2 \theta$ (1, Table 12) in order to stabilize the variance for statistical analysis. Table V shows the analysis of variance of these transformed quantities. The effect of initial treatment reached the level of statistical significance for both external mould growth and internal spoilage. In addition significant differences in internal spoilage were observed between replicate half cases. Tray positions within the case had little effect. A statistical analysis of the relation of position in the tray to both external and internal spoilage showed no significant difference in either respect between eggs at the outside of the trays and those on the inside.

The amount of external mould on unhandled producer eggs was less than on those subjected to all other treatments (Table III). Individually these differences were statistically significant for all except untreated market eggs in which the difference almost reached significance. Oil dipped eggs and those treated with hydrogen peroxide followed by an oil dip had more external mould growth than those of any of the other treatments and the differences were statistically significant except between the hydrogen peroxided oil dipped eggs and producer eggs handled in the ordinary manner. Untreated and contaminated market eggs and ordinarily handled producer eggs showed only small differences among themselves in the average amount of external mould growth.

The average percentage of internal spoilage at the end of the storage period was markedly less in producer eggs than in any of the four categories of market eggs (Table IV), and these differences were statistically significant. Although unhandled eggs had the least internal spoilage the difference between the producer egg treatments was very slight. In the market eggs the greatest average spoilage occurred in the contaminated lots and statistical analysis showed the difference between this and the untreated to be significant. While the observed spoilage in the oiled eggs was somewhat greater than in the untreated, the difference was not statistically significant. A progressive increase occurred in the half-case average percentage internal spoilage which was statistically highly significant.

Discussion

There seems to have been no clear cut relation between the number of moulds originally present on the shells of the eggs and their subsequent growth on the surface during storage. Under the severe storage conditions of this experiment, however, it is evident that oil dipping and handling both favoured the growth of exterior moulds. That mould growth is greater on oiled than on unoiled eggs after storage at temperatures and humidities approximating the severest summer conditions, is in agreement with previous work (5).

Since only one of the original 36 groups of eggs showed infection in the egg meats, it seems reasonable to conclude that most of the organisms causing interior spoilage at the end of the storage period entered from the outside. The significant differences found between the spoilage of producer unhandled (low count) and market untreated (medium count) eggs and between untreated and contaminated (high count) market eggs suggest that the amount of shell contamination may play an important part in internal egg spoilage, but since treating market eggs with hydrogen peroxide (very low count) before oil dipping made little difference to either exterior mould growth or interior spoilage, it seems possible that shell penetration by micro-organisms in this instance had proceeded to the point where surface disinfection had little value. On the other hand in spite of the fact that producer ordinarily handled eggs had heavy exterior mould growths, the amount of internal spoilage was small which indicates that fresh egg might be bacteriostatic and mouldistatic.

The progressive increase in internal spoilage in each half-case lot (Table IV), the only difference in treatment being the time each case was stored under alternating and constant conditions of temperature and humidity (Table I), suggests that interior spoilage is hastened when both temperature and humidity are high. This observation is substantiated by other investigations (2, 4).

The wearing of clean dry gloves whenever eggs are to be handled is a practice which can be recommended. There is no doubt that a reduction in egg spoilage losses could be effected by a more rigid application of recognized sanitary practices both by the producer and in the trade.

Acknowledgments

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PRESERVATION OF EGGS

III. STUDIES ON THE EFFECT OF OIL TREATMENT AND EGG-CASE LINER BAGS IN THE PRESERVATION OF SHELL EGGS UNDER ADVERSE CONDITIONS[1]

By E. J. Reedman[2] and J. W. Hopkins[3]

Abstract

Three replicate lots of eggs, each divided into half-case (15 doz.) sets on the treatments used, were stored at 70° F. and 90% relative humidity for 35 days. Different sets of eggs were conditioned at 32°, 40°, 60°, and 70° F. before being placed in the humidity chamber, but no resulting effect of initial condensate on the eggs and packaging material was demonstrated.

Under the conditions of this experiment, oil treatment by the best commercially applicable method, followed by packaging in sealed egg-case liner bags, was found to retard greatly the development of internal defects and severe external mould. Oil treatment alone was definitely beneficial, but the use of egg-case liner bags on untreated eggs was detrimental. While these experiments show that the method of oil treatment combined with bagging was efficacious in these laboratory scale tests, the results obtained from three small test shipments to Great Britain were not in agreement, and indicated that somewhat different storage conditions may be encountered in the export of shell eggs in non-refrigerated holds. The length of time of storage may also be expected to influence directly the degree of spoilage.

Differential spoilage of eggs located in the central and top and bottom trays within the case, and at the periphery and in the interior of individual trays, was also noted under certain conditions. This is considered to indicate the importance of humidity gradients within the case.

Introduction

In the storage and marketing of perishable foods such as shell eggs, a major consideration is protection against spoilage by micro-organisms. The natural defences of shell eggs are not sufficient to prevent spoilage when they are subjected to conditions of temperature and humidity favourable for the growth of moulds and bacteria. The object of this investigation was to make a laboratory study of the efficacy of three commercially practicable treatments which might be used to inhibit spoilage and conserve quality attributes in shell eggs handled under adverse conditions.

The treatments used were oil dipping, using a type of oil and method of application known to be most satisfactory for the test and conditions, oil treatment used in conjunction with packaging in sealed egg-case liner bags, egg-case liner bags used alone with untreated eggs, and untreated eggs as controls. A factor considered to contribute to spoilage in eggs trans-shipped commercially from a cooler to a warmer atmosphere, is the formation of condensate when the humidity is high. Each treatment was accordingly tested under four simulated "handling conditions", giving a graded series of

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degrees of condensate on the surface of the eggs and packaging material when trans-shipped to the storage room. The temperature and relative humidity of the storage room were maintained at 70° F. and 90%, respectively, throughout the experiment.

While treatment of shell eggs by immersion in oil, and subsequent drainage of excess oil prior to packaging, has been used to a considerable extent in commercial storage (3, 4), little information is available on the effect of oil treatment at higher storage temperatures. The oil employed in these experiments was a low pour-point paraffin base mineral oil having a Saybolt viscosimeter number of 50/60 at 100° F. This type of oil has been used in commercial storage, and experiments had shown it to be generally superior to heavier oils and waxes for the purpose of this study. The egg-case liner bags used were constructed of two layers of Kraft paper laminated with asphalt, and all were sealed to restrict contact of outside air with the contents.

Experimental

Source of Eggs

Three replicates of the experimental treatments were made, using Grade A Large eggs secured from three central grading stations during the month of April. Grading included candling to obtain as uniform experimental material as possible. White-shelled eggs were used throughout to eliminate the necessity of distribution as to shell colouring in the test lots. Shell porosity could not be measured objectively, but it was possible to choose eggs as nearly alike as could be judged by shell formation. None of the eggs obtained were more than 24 hr. old.

Mould and bacterial load of the eggs as received could not be determined by any commercially practicable method, but variation was minimized by obtaining eggs as nearly new-laid as possible. In view of the fact that all eggs were secured from the grading stations within 24 hr. of their delivery from nearby farms, it is possible that the degree of mould infection at the time of treatment was lighter (2), and the test of treatment correspondingly less severe, than would normally be the case in commercial practice. It is generally realized that oiling or other similar methods of treatment must be applied to fresh eggs subjected to no major contamination, unless some reduction in the microbial flora of older eggs is feasible.

Treatment

After grading, the eggs selected for experimentation were immediately transported to the laboratory by truck, and were conditioned to laboratory temperature before treatment. By use of commercially available egg lifting trays the eggs were removed from the cases, oil dipped, drained, and replaced without touching by hand. Untreated eggs were repacked after conditioning to the same degree as treated eggs.

As earlier findings had indicated decreased penetration of oil when using cool eggs dipped in a heated oil, it was found most convenient to condition all eggs to 80° F. and to dip them in oil conditioned to the same temperature. Under these conditions a 10 sec. immersion and five-minute drainage time (which could be reduced to one minute in commercial practice) were found by experimentation to be adequate, and were adopted throughout. The "small ends" of the eggs were wiped over a brush to remove any excess oil which would not drain off the eggs on standing in the egg lifting tray.

Preliminary tests were made to determine whether treating successive lots of eggs with the same egg dipping oil would result in the oil becoming infected with organisms. After dipping 120 doz. eggs in a 3 gal. volume of oil there was found to be no appreciable increase in microbial content as compared to unused oil. Examination for moulds and bacteria was made by 70° F. incubation of 1 ml. samples with beef peptone and potato dextrose agars for five and four days respectively.

The egg cases used were of the standard ventilated export type having a capacity of 30 doz. The experimental unit was 15 doz. placed in one compartment of each case. To eliminate any variation caused by contamination by fillers and cases, these were sterilized before use. Commercial Keyes trays were used throughout in packaging. In all, three replicates of four experimental sets through four handling procedures required the use of 48 half-case lots of eggs. Each treatment was thus tested on 12 half-case lots under the total of four handling procedures.

Handling and Storage

After treatment, the eggs (including control eggs and untreated eggs in egg-case liner bags, which were simply repacked) were placed in the conditioning chambers prior to storage in the humidity room at 70° F. One half-case set of each treatment in each replicate lot was conditioned to 32° F., one to 40° F., one to 60° F., and one to 70° F. After conditioning, the eggs were moved immediately, through a cool dry atmosphere, to the insulated storage test room at 70° F. and 90% relative humidity. In this way graded amounts of condensate, from a maximum to a minimum were obtained initially on the shells of the eggs and on the fillers and cases.

The storage room was stacked full of cases at all times, empty cases being used as necessary to ensure a constant condition in this regard. The room was ventilated by an extremely slow movement of air through the floor, and the cases containing the experimental eggs were stacked in an objectively random order. Material from previous experiments on egg storage had resulted in a high degree of mould contamination in the storage room, but

plates of potato dextrose agar, exposed in various parts and at various levels, showed a uniformity of mould distribution in the air in which the eggs were stored.

Several pilot half-case lots receiving the various treatments were stored at the same time and examined at intervals. This permitted the gauging of the progress of deterioration. The experimental eggs in the three replicate series were examined only once, after a storage period of 35 days. Thus contamination caused by disturbing the eggs was eliminated, while observations on the pilot lots were indicative of a condition suitable for comparison of treatment.

Examination

The experimental eggs were first examined individually for evidence of external mould growth, and then candled for internal mould and spot rot. The incidence of each type of spoilage was noted separately, and graded visually as either "mild" or "severe". The location and condition of every egg in each tray was recorded on a mimeographed diagram.

External examination was made in a strong light which enabled the detection of very mild growth such as the "whisker" growth of *Mucor*.

Internal moulds show up readily on candling, and may appear as minute black, green, or red spots, or may cover an appreciable area inside the shell. In the present investigation, many instances of mould inside the air cell were found. In some eggs it was difficult to determine if an observed small spot was actually due to mould growth, or was merely a speck of foreign material within the layers of the shell. Such eggs were broken open and a careful examination made.

Moulds do not normally penetrate into the yolk of the egg except in advanced stages of deterioration. Yolk spoilage is generally caused by bacterial growth, sometimes known as spot rot. Spot rot may vary in degree from small spots to a condition turning the whole interior of the egg into a putrid mass. For the purpose of this study the degree of spot rot was taken as "mild" when a spot of the most minute character could be distinguished. Brown and black *Proteus* rots were observed to be most common, but red *Pseudomonas* also occurred.

Results

The design of the experiment was such that all observed percentages could be treated statistically by the analysis of variance procedure, and the significance of any apparent difference was therefore assessed in this way. A

primary analysis was made of the effect of handling and treatment on half-case aggregates, and a secondary analysis of the extent to which treatment effects were modified by the location of the eggs within the half-case.

Lot Averages

Table I shows the average percentage of defects observed at the conclusion of storage in eggs from each of three sources. Significant differences, particularly in respect of internal contamination, are evident. As was noted above, however, treatment comparisons are balanced in respect of such average differences, which may therefore be regarded as an advantage in that they provide a broader basis for the treatment averages.

TABLE I

PERCENTAGE INCIDENCE OF DEFECTS IN EGGS FROM THREE SOURCES AFTER STORAGE FOR 35 DAYS AT 70° F. AND 90% R.H.* (AVERAGE OF ALL TREATMENTS AND HANDLING CONDITIONS)

Defect	Source I	Source II	Source III
External mould, mild or severe	97.9	99.8	100.0
External mould, severe	38.1	42.3	45.8
Internal mould, mild or severe	27.1	35.4	47.6
Internal mould, severe	1.2	10.1	22.4
Spot rot, mild or severe	3.6	6.1	8.8
Any internal defect (mould, spot rot, or both)	30.5	40.9	55.0

* *R.H. = relative humidity.*

Effect of Handling Conditions on Half-case Aggregates

Under the conditions of this experiment the imposed differences in handling procedure had no statistically significant effect on the percentage of external mould, internal mould, or spot rot at the conclusion of storage in either treated or untreated half-case aggregates. The average percentages of severe external mould observed in all the half-cases conditioned at 32°, 40°, 60°, and 70° F. were 47, 42, 35, and 44% respectively, whilst the corresponding figures for internal defects (mild or severe) were 47, 42, 41, and 39%. It is possible that the high temperature and humidity of the storage room resulted in too severe a condition to allow marked differences between the effects of various amounts of condensate.

Effect of Oiling and Bagging on Half-case Aggregates

The percentages of defects recorded after 35 days' storage following each treatment are set forth in Table II. These percentages are each computed from 12 half-case lots (three replicates of each of four handling conditions) containing a total of 180 doz. eggs.

TABLE II

PERCENTAGE INCIDENCE OF DEFECTS IN EGGS AFTER STORAGE FOR 35 DAYS AT 70° F. AND 90% R.H. FOLLOWING VARIOUS TREATMENTS (AVERAGE OF THREE REPLICATIONS OF EACH OF FOUR HANDLING CONDITIONS)

—	Control (untreated)	Oil only	Bags only	Oil and bags
External mould, mild or severe	98.3	99.9	100.0	98.6
External mould, severe	40.9	30.3	96.1	1.1
Internal mould, mild or severe	51.2	30.8	63.5	1.2
Internal mould, severe	15.8	2.2	26.8	0.1
Spot rot, mild or severe	10.7	1.1	12.8	0.1
Any internal defect (mould, spot rot, or both)	58.5	31.6	77.2	1.3

Practically all eggs showed some external mould, either mild or severe, irrespective of treatment. The incidence of severe external mould was, however, significantly affected by oiling and bagging. Oiling without bagging caused a reduction of about 10% in comparison with the untreated controls. Oil treatment in the absence of a mycostatic agent cannot be expected to reduce the incidence of external mould appreciably, and its effect is conditioned by the type of eggs and the conditions of storage (2). Bagging without oiling clearly favoured growth, and led to an increase of 55% in severe external mould. On the other hand the combination of oiling with bagging proved to be very effective, and restricted severe external mould to about 1% of the eggs treated in this way.

Total internal mould (mild or severe) was reduced by about 20% following oiling alone, but was appreciably further restricted (to about 1%) by the combination of oiling and bagging. Bagging without oiling was markedly detrimental. Similar tendencies manifest themselves in the recorded percentages of severe internal mould, although oiling alone was practically as effective as oiling and bagging. Spot rot was likewise effectively inhibited by oiling, but was not significantly affected by bagging either alone or in conjunction with oiling.

Observations based on size of air cell, yolk index, and hydrogen ion concentration of the white showed that at the conclusion of storage the average quality of the untreated eggs and those bagged without oiling was poor. That of the oiled eggs was considered to be good, and that of those oiled and bagged very good.

Influence of Position in Half-case

As was mentioned above, practically all eggs developed some degree of external mould. There were, however, appreciable differences in the incidence of severe external mould in both untreated and oiled eggs ascribable to location within the case, as illustrated in Table III. Both these types of eggs were subject to less severe external mould in the top and bottom trays than those in the middle of the case. On the other hand the percentage of this defect in eggs bagged without oiling was uniformly high in all trays, and in those both oiled and bagged, uniformly low.

TABLE III

PERCENTAGE OF SEVERE EXTERNAL MOULD AFTER STORAGE FOR 35 DAYS AT 70° F. AND 90% R.H. IN EGGS CLASSIFIED ACCORDING TO TREATMENT AND LOCATION IN CASE (AVERAGE OF THREE REPLICATIONS OF EACH OF FOUR HANDLING CONDITIONS)

Location	Control (untreated)	Oil only	Bags only	Oil and bags
First tray (top)	11.1	30.9	93.4	3.9
Second tray	49.6	37.6	96.4	0.6
Third tray	55.3	39.0	97.5	1.4
Fourth tray	53.5	41.0	98.6	0.0
Fifth tray	48.0	26.7	96.9	0.3 Nec. diff.
Sixth tray (bottom)	16.0	8.0	96.1	0.3 = 9.2
Inside rows (all trays)	56.0	40.2	99.5	1.7 Nec. diff.
Outside row (all trays)	27.5	24.1	94.3	0.6 = 5.4

The controls and the eggs oiled without bagging also exhibited a lower frequency of severe external mould if located in the outside row, rather than in the central portion of a Keyes tray. This differential varied somewhat from tray to tray, but was appreciable in the untreated eggs subjected to all four "handling" temperatures (Table IV), whereas in oiled eggs it was definitely most pronounced following "handling" at 32° F., and was statistically insignificant in the cases handled at 60° F., and 70° F. Severe external mould was

TABLE IV

PERCENTAGE OF SEVERE EXTERNAL MOULD AFTER STORAGE FOR 35 DAYS AT 70° F. AND 90% R.H. IN EGGS "HANDLED" AT 32°, 40°, 60°, AND 70° F. (AVERAGE OF THREE REPLICATIONS)

——	32° F.	40° F.	60° F.	70° F.
Untreated				
Inside rows (all trays)	67.9	44.4	45.7	66.2
Outside rows (all trays)	32.1	25.9	19.5	32.7
Oil only				
Inside rows (all trays)	70.6	38.9	14.4	36.9 Nec. diff.
Outside row (all trays)	34.0	24.2	8.9	29.3 = 10.8

uniformly high in both the inner and outer rows of trays of eggs bagged without oiling, and in those both oiled and bagged it was uniformly infrequent irrespective of position.

Internal defects were likewise less frequent in the bottom trays of the cases containing eggs untreated, oiled only, or bagged without oiling, as indicated in Table V. The untreated eggs also showed fewer of these defects in the top

TABLE V

PERCENTAGE OF INTERNAL DEFECTS (MILD OR SEVERE MOULD, SPOT ROT, OR BOTH) AFTER STORAGE FOR 35 DAYS AT 70° F. AND 90% R.H. IN EGGS CLASSIFIED ACCORDING TO TREATMENT AND LOCATION IN CASE (AVERAGE OF THREE REPLICATIONS OF EACH OF FOUR HANDLING CONDITIONS)

—	Control (untreated)	Oil only	Bags only	Oil and bags	
First tray (top)	55.0	35.8	68.1	2.3	
Second tray	59.6	30.8	71.3	2.0	
Third tray	65.0	30.5	69.2	0.6	
Fourth tray	64.4	34.3	68.0	1.1	
Fifth tray	61.4	35.4	64.9	0.6	Nec. diff.
Sixth tray (bottom)	44.3	23.4	61.7	0.9	= 6.6
Inside rows (all trays)	67.5	30.5	85.5	1.4	Nec. diff.
Outside rows (all trays)	52.2	32.5	55.0	1.1	= 3.8

than in the middle trays, but this is not true of the treated eggs. On the average there was a significantly higher percentage of internal defects in control eggs and in those bagged without oiling when located in the interior of the tray than when at the outside (Table V). This effect was, however, a function of "handling" temperature, and is accordingly analysed in more detail in Table VI. From this tabulation it is to be seen that the controls

TABLE VI

PERCENTAGE OF INTERNAL DEFECTS (MILD OR SEVERE MOULD, SPOT ROT, OR BOTH) AFTER STORAGE FOR 35 DAYS AT 70° F. AND 90% R.H. IN EGGS "HANDLED" AT 32°, 40°, 60°, and 70° F. (AVERAGE OF THREE REPLICATIONS)

—	32° F.	40° F.	60° F.	70° F.	
Untreated					
Inside rows (all trays)	73.1	71.4	61.2	64.3	
Outside row (all trays)	56.3	52.5	49.7	50.2	
Oil only					
Inside rows (all trays)	50.4	34.2	19.7	17.7	
Outside row (all trays)	41.8	31.3	31.1	25.9	
Bags only					
Inside rows (all trays)	80.7	85.3	88.4	87.6	
Outside row (all trays)	70.5	45.9	53.0	50.7	
Oil and bags					
Inside rows (all trays)	4.2	0.0	1.3	0.0	Nec. diff.
Outside row (all trays)	2.6	0.0	1.6	0.3	= 7.6

showed such a differential following all four handling temperatures. So did the eggs bagged without oiling, but here the difference was definitely less pronounced in those cases handled at 32° F. than in those subjected to any of the other three handling temperatures. It is also noteworthy that the eggs oiled without bagging showed more defects at the outside than in the interior of the trays following handling at 32° F., but less after handling at 60° and 70° F.

Discussion

It is evident from Table I that different lots of eggs may develop appreciably different amounts of spoilage during storage. The three lots of eggs were all taken at one season of the year, and indicate variation between eggs gathered from different areas throughout a small section of Ontario. Further variation between seasons has been observed in other experiments (1). Since the amount of spoilage may be determined to a considerable extent by contamination prior to treatment and storage, the success of oil treatment in practice may well depend on its application early to relatively uncontaminated eggs (2).

Oil treatment of shell eggs prevented spoilage to an appreciable extent, but was not as effective as oil treatment followed by packing in egg-case liner bags under the conditions of this test. Bagging without oiling was clearly detrimental, probably because of saturation of the atmosphere within the bags by water vapour diffused from the eggs themselves. Oil treatment, if properly applied, retards the loss of moisture from eggs and this was possibly responsible for the difference obtained in the use of the egg-case liner bags.

Some indication of the effect of physical conditions on the development of micro-organisms may be seen from Tables III to VI. The imposed differences in prestorage handling temperature were designed to illustrate the effect of condensate from the external atmosphere. The results show that the degree of condensate was apparently not the main factor responsible for the differential spoilage of eggs at the centre and periphery of the cases, as this occurred in the absence of condensate. It clearly had some effect in modifying this differential in eggs which were not enclosed in egg-case liner bags. Maintenance of a steady storage temperature is considered to have favoured the bagging treatments by preventing condensation after storage for a length of time in which moisture could have accumulated within the bags.

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PRESERVATION OF EGGS

IV. STORAGE OF OILED AND BAGGED EGGS UNDER CONSTANT AND ALTERNATING TEMPERATURES AND HUMIDITIES[1]

By N. E. Gibbons[2], C. O. Fulton[2], and J. W. Hopkins[3]

Abstract

Untreated eggs and eggs oiled and sealed in egg-case liner bags were stored for six weeks in standard export cases (*a*) at 70° F. and 90% relative humidity continuously, (*b*) at 65° F. and 95% relative humidity alternating with 75° and 70% every two days, the dew point consequently always remaining below 65° F., and (*c*) at 60° F. alternating with 80° every three days, both at 90% relative humidity, but the dew point varying from 57° to 77° F. respectively. Spoilage was least under the second set of conditions and greatest under the third. The indications were that within rather wide limits, microbiological development was dependent primarily upon storage conditions rather than upon the quantity of inoculum on the surface of the eggs at the beginning of storage. The bags used had little effect on intracase temperatures.

Under conditions (*a*) and (*c*) oiling and bagging reduced internal mould and rot but did not significantly affect the development of external mould; under conditions (*b*) internal spoilage was uniformly low in both treated and control eggs. All oiled and bagged eggs were in better physical condition after storage than were the untreated, having smaller air cells, freer yolks, and less distinct yolk shadows. Variations in the incidence of spoilage within cases occurred and are attributed to intracase temperature and humidity differentials.

Introduction

In a previous study in these laboratories (1) it was observed that oiling followed by enclosure in egg-case liner bags appreciably reduced mould and bacterial spoilage of eggs stored for six weeks at 70° F. and 90% relative humidity. Subsequent trial overseas shipments were not, however, productive of similar findings, from which it appeared that the foregoing experimental storage conditions might not be a suitable substitute for those typically encountered in shipping. The two additional experiments now reported were accordingly undertaken in order to accumulate further information respecting the influence of environmental conditions on the efficacy of this mode of treatment of shell eggs.

In the first of these, treated and control eggs characterized by three levels of initial bacterial and mould contamination of the shell were subjected to cyclic variations in temperature and humidity in which two days at 65° F. and 95% relative humidity alternated with two days at 75° F. and 70% relative humidity successively for six weeks. The relative humidity was thus higher in the low phase of the temperature cycle, and *vice versa*, but the dew

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[3] *Statistician.*

point remained practically constant. In the second test a temperature differential of 20° F. was imposed, three-day periods at 60° alternating with three days at 80° F., again for six weeks. The relative humidity was maintained at the high level of approximately 90% throughout and consequently the dew point varied from 57.2° F. to 77° F. at temperatures of 60° and 80° F. respectively. For comparative purposes, the previously employed constant storage conditions of 70° F. and 90% relative humidity were also repeated in both tests.

Experimental

Eggs

All eggs used were obtained from one eastern Ontario grading station in January and April. White-shelled Grade A Medium eggs were specified, but for the first experiment it was necessary to accept four cases (out of 18) of Grade A Pullet eggs. The contents of all cases were accordingly redistributed so as to include whole trays of pullet eggs in random order in each case. Subsequent analyses of variance showed that these did not differ significantly from the medium eggs in respect of either internal or external spoilage.

Three replicate lots each comprising six 30-doz. cases or 12 15-doz. experimental units were employed in the first experiment. In the second experiment three lots of three cases each were used. These were disposed so as to provide six replicates of each 15-doz. experimental unit under alternating and three under constant storage conditions.

Shell Contamination

In the first experiment three levels of initial shell contamination were introduced. To provide these, two-case lots of each replication were dipped in a disinfectant solution of 3% U.S.P. hydrogen peroxide containing $\frac{1}{5}$ gr. acetanilid per oz., two were left untreated, and the remaining two were dipped in a saline suspension of bacteria and fungi. This last contained a mixture of representative types of organisms isolated from bad eggs, growths from two-day old plate cultures of bacteria and week-old cultures of fungi being washed off and diluted with saline solution to give a just cloudy suspension. Bacterial counts at the time of dipping the first, second, and third replications were 3,500,000, 2,100,000, and 66,500,000 per ml. respectively. Before the dipping of the second replication the mould count was 150,000 per ml., but no moulds appeared in a 1 : 10,000 dilution made prior to dipping the third replication. In the interval between dipping replicates, the suspension was maintained at 45° F., but on each occasion was brought into the laboratory the night before being used and was allowed to come to room temperature.

By means of commercially available egg lifters, tray lots were dipped simultaneously, the eggs being immersed for one minute and drained for 30 sec. After draining, peroxide dipped eggs were transferred at once to sterilized Keyes trays and left at laboratory temperature. Those dipped in the suspension of organisms were returned to their original trays and while

still wet placed in a room maintained at 30° F., on the assumption that sudden chilling might draw bacteria and mould spores through the shell. Four to five hours later they were returned to the laboratory and left along with the peroxide dipped and untreated eggs until the next day, when half of each case lot was oiled and bagged.

Throughout this period, laboratory temperature was about 25° C. (77° F.).

Oiling and Bagging

All eggs in both experiments were left at laboratory temperature overnight prior to the oiling and bagging of half of each case (selected at random). Control (unoiled and unbagged) eggs were transferred to sterile Keyes trays where necessary and placed in the appropriate half of a sterilized export case. In the first experiment oiling and packing were done in the order: peroxided, untreated, and contaminated. In both experiments, eggs were dipped for 10 sec. in a mineral oil of the same composition as used previously (1) (fresh oil being used for each replication) and drained for at least three minutes. The drop of oil remaining on the small end of each egg after drainage was removed with an oiled brush, following which bagging took place immediately. The bags used were of the asphalt laminated egg-case liner type employed previously, the tops of which were folded over and securely fastened with gummed paper and cellulose tape.

Storage

One-half of the eggs (nine cases) in the first experiment and one-third (three cases) of those in the second were again kept under the constant storage conditions previously employed, whilst the remainder were subjected to alternating temperatures and humidities designed to simulate and even to exaggerate changes during transport. Once all cases had been placed in the storage rooms the position of each was changed every four days during the first experiment according to a random scheme, the cases being turned end for end with each change. During the second experiment, the position of the cases was changed every week.

During the first experiment, the average temperature over the whole storage period in the constant condition room was 70.5° F. and the average relative humidity, 91.5%. The highest temperature recorded was 72.5°, but on two occasions readings of 60.5° and 61.5° F. respectively, occurred as a result of pump and fan failures. Relative humidity fluctuations were confined to the range 83 to 95%. In the alternating condition room the change from one set of conditions to the other was usually completed within

three to four hours (Fig. 1). There were greater uncontrolled fluctuations in this than in the former room, but the recorded extremes of temperature and humidity were 59° and 86° F., and 58 and 100% relative humidity, respectively. The averages, over the whole experiment, of the first phase of the

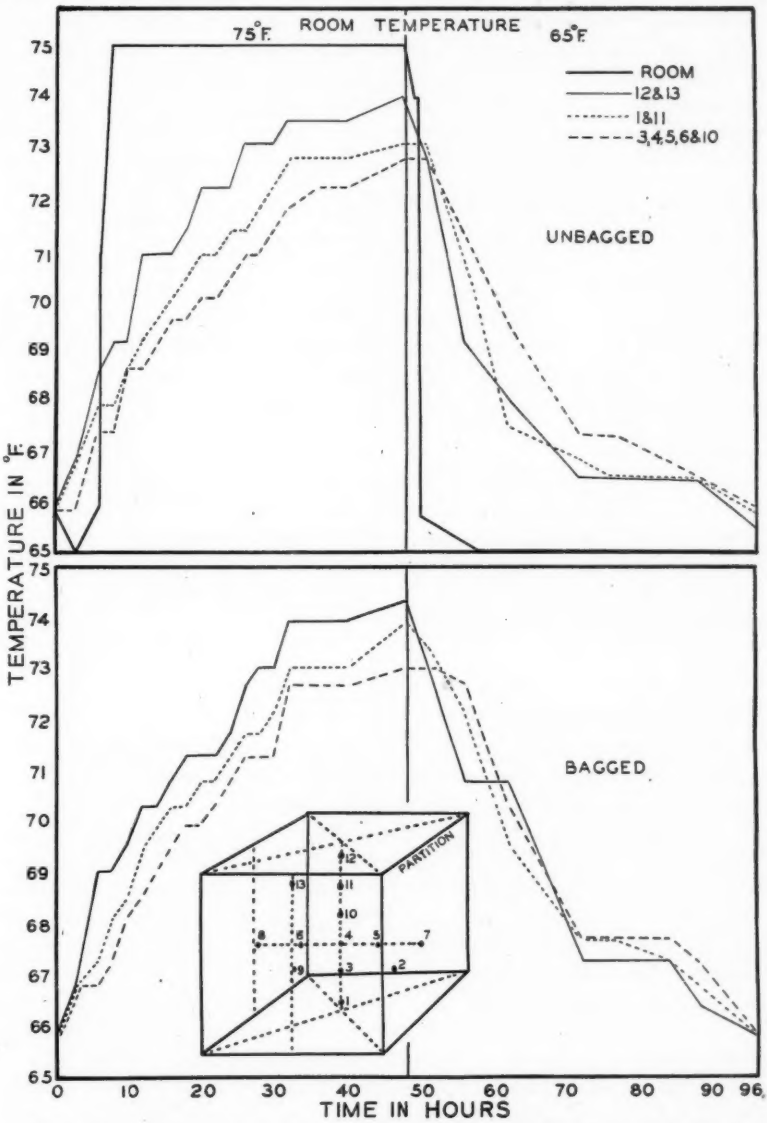

FIG. 1. *Change of temperature in egg cases following changes in external temperature from 65° to 75° F.*

temperature and humidity cycle were 63.7° F. and 95.7% relative humidity, and of the second 74.4° F. and 69.6% relative humidity.

In the second experiment, the averages for the constant condition room were 70.7° F. and 92.4% relative humidity. The average temperature in the high phase of the cycle in the alternating room was 80.0° F., but there were greater irregular fluctuations than previously, and a minimum of 50° and a maximum of 110° F. were recorded for short periods. The average temperature for the low phase was 62.4° F., although again extremes of 45° and 97° were recorded for brief intervals. Relative humidity was on the average 87.3% at the high and 86.1% at the low temperature.

Measurements of Intracrate Temperature

To secure measurements of intracrate temperatures, copper-constantan thermocouples were installed in one of the crates stored in the alternating room during the first experiment. These were fastened with tape to points on the various trays as indicated in Fig. 1, corresponding points being selected on each side of the central position, i.e., in the bagged and unbagged half of the crate. In the former, the thermocouple leads were brought out through the top of the bag and the bag sealed with hot asphalt.

Examination of Eggs

After six weeks of storage all eggs were individually examined under a strong light for signs of external mould, and were then candled (and in any doubtful instance broken out) for the recording of internal defects. No attempt was made to distinguish between internal mould and rots. Stuck yolks, jelly spots, and tiny mould colonies were recorded as mild, and all definite mould colonies and rots as severe.

Results

Intracrate Temperature Alternations

Observations were made at intervals over two complete temperature cycles when the experimental crate was in different positions in the room, but as the results for both were similar, the changes during one cycle only are shown in Fig. 1. As would be anticipated there was an appreciable lag in both the uptake and loss of heat by the crate, and actually the internal temperature in both the bagged and unbagged portion did not quite reach equilibrium with that of the surrounding air before the next phase of the cycle began. The bag appeared to exert no appreciable effect on the rate at which the internal temperature rose, but retarded cooling somewhat. In both the bagged and unbagged half of the crate the first (top) tray responded most rapidly to external changes, and the second and bottom ones were next, while the middle trays showed the greatest lag. The central group of thermocouples (3, 4, 5, 6, and 10 in Fig. 1) usually exhibited a range of less than 1° F. No. 13, located at the edge of the top tray, was usually about half a degree ahead of No. 12 (top centre), but this differential was not observed in the middle and lower

parts of the crate. The two top trays most nearly approached room temperature, coming to within about a degree of the upper and about half a degree of the lower level. In the case of the other trays the discrepancy was of the order of one degree at the termination of both the high and low phases.

TABLE I

AVERAGE PERCENTAGE OF EGGS SHOWING EXTERNAL MOULD AND INTERNAL DEFECTS (FIRST EXPERIMENT)

Treatment	Severe		Mild + severe	
	65° F., 95% R.H.* and 75° F., 70% R.H. alternating	70° F., 90% R.H. continuously	65° F., 95% R.H. and 75° F., 70% R.H. alternating	70° F., 90% R.H. continuously
	External mould			
Oiled and bagged	0	19.1	2.5	59.1
Control	0	11.3	1.9	68.7
	Internal defects			
Oiled and bagged	2.6	2.7	4.5	21.8
Control	4.5	51.6	6.2	66.6

**R.H.=relative humidity.*

External Mould in Half-case Aggregates

The average percentages of external mould recorded under the various conditions of the first experiment are shown in Table I, whilst Table II summarizes the results of an analysis of variance of the data for individual half-cases.

TABLE II

ANALYSIS OF VARIANCE OF PERCENTAGE OF EXTERNAL MOULD AND INTERNAL DEFECTS IN EGGS STORED UNDER CONSTANT AND ALTERNATING CONDITIONS (FIRST EXPERIMENT)

Source of variance	D.f.	Mean square, exterior mould		Mean square, interior defects	
		Severe	Mild + severe	Severe	Mild + severe
Lots	2	1176.84*	3450.48**	731.89	2126.51*
Shell contamination	2	197.69	698.79	601.51	1042.43
Storage conditions	1	2076.32**	34249.67**	4998.49**	13564.48**
Treatment	1	136.11	180.45	5816.61**	4867.39**
Shell × storage	2	197.70	202.60	321.50	545.82
Shell × treatment	2	34.23	350.06	291.55	129.55
Storage × treatment	1	136.11	233.07	4974.95**	4177.47**
Shell × storage × treatment	2	34.22	342.39	362.44	270.64
Error	22	260.29	470.27	284.46	343.49

* *Indicates 5% level of significance.*
** *Indicates 1% level of significance.*

The constant conditions of 70° F. and 90% relative humidity were much more favourable for the growth of mould on the outside of the shells than were the alternations of 65° and 95% and 75° and 70%. Under the latter in fact no severe and only a slight amount of mild external mould developed. There was a statistically significant difference between the three replicate lots of eggs, the third being most susceptible to spoilage of all kinds, but the design of the experiment was such that this affected all treatment comparisons equally. Oiling and bagging was without apparent effect on the development of external mould under either set of conditions, nor was any influence of the level of initial shell contamination demonstrable.

TABLE III

AVERAGE PERCENTAGE OF EGGS SHOWING EXTERNAL MOULD AND INTERNAL DEFECTS (SECOND EXPERIMENT)

	Severe		Mild + severe	
	60 – 80° F., 90% R.H.	70°F., 90% R.H. continuously	60 – 80° F., 90% R.H.	70° F., 90% R.H. continuously
	External mould			
Oiled and bagged	15.0	2.4	76.9	94.6
Control	30.9	1.7	93.0	77.4
	Internal defects			
Oiled and bagged	9.1	1.5	24.1	8.3
Control	80.5	44.4	96.0	72.8

Since in the second experiment only three cases were stored under constant and six under alternating conditions, separate analyses of variance of the two series of results are given in Table IV. Following the alternating conditions

TABLE IV

ANALYSIS OF VARIANCE OF PERCENTAGE OF SPOILAGE IN EGGS STORED UNDER ALTERNATING AND CONSTANT CONDITIONS (SECOND EXPERIMENT)

Source of variance	D.f.	Mean square, external mould		Mean square, internal defects	
		Severe	Mild + severe	Severe	Mild + severe
Alternating conditions					
Replicates	5	1102.6*	293.3*	164.7	252.6
Treatments	1	761.6	776.0**	15279.6**	15508.8**
Error	5	136.3	45.4	77.7	180.5
Constant conditions					
Replicates	2	0.882	151.2	151.3	11.4
Treatments	1	0.807	443.8	2769.2*	6233.9*
Error	2	9.882	48.7	134.8	121.8

* *Indicates 5% level of significance.*
** *Indicates 1% level of significance.*

of this test there was significantly less external mould on the oiled and bagged than on the control eggs, but under the constant conditions the observed differences between treatments were not statistically significant considering the small number of cases employed (Table III).

Internal Defects in Half-case Aggregates

When the eggs were being candled after storage it was noted that the oiled and bagged ones were for the most part in better internal condition than the controls, having smaller air cells, freer yolks, and less distinct yolk shadows. Of the "gradeable" eggs, most of those oiled and bagged would grade B, whilst the unbagged ones were of Grade C.

In the first experiment (Tables I and II) the incidence of internal defects under the alternating conditions imposed was uniformly low irrespective of the level of initial shell contamination and of whether the eggs were oiled and bagged or not. Under the constant conditions however, much more internal spoilage developed in the control than in the oiled and bagged eggs. As in the case of external mould, there were significant differences in the average amount of spoilage in the replicate lots of eggs, but again no significant effect of the artificial modifications of initial shell contamination although the observed amount of spoilage increased in the order: peroxided, control, and contaminated eggs.

A beneficial effect of oiling and bagging in reducing internal spoilage was also observed in the second experiment (Tables III and IV), but in this case following alternating as well as constant storage conditions.

Incidence of Spoilage in Relation to Position of Egg in Case

From the previous study (1) there was some indication of differential spoilage of the eggs located on the outside and inside rows of the Keyes trays. In considering this point in the present investigation, eggs adjacent to an end or side of the crate were classified as "outside", whilst those adjacent to the central partition were classed together with those in the central portion of the trays as "inside".

Table V gives the average percentages of inside and outside eggs showing spoilage in the first experiment. Under the alternating storage conditions of this test the incidence of both external and internal growths was uniformly low in both oiled and bagged and control eggs, irrespective of their position in the tray. Under constant temperature and high humidity conditions on the other hand a more frequent occurrence of both external and internal spoilage in the central portion of the trays was statistically demonstrable for control eggs, whilst the effect of oiling and bagging was not only to reduce the total amount of deterioration as already noted, but also to eliminate the foregoing differential between the inside and outside rows of the trays.

TABLE V

AVERAGE PERCENTAGE OF SPOILAGE IN INSIDE AND OUTSIDE ROWS OF TRAYS (FIRST EXPERIMENT)

	Severe		Mild + severe	
	65° F., 95% R.H. and 75° F., 70% R.H. alternating	70° F., 90% R.H. continuously	65° F., 95% R.H. and 75° F., 70% R.H. alternating	70° F., 90% R.H. continuously
	External mould			
Oiled and bagged				
Inside	0	18.0	3.5	59.4
Outside	0	20.2	1.5	58.9
Control				
Inside	0	14.8	3.5	73.1
Outside	0	7.5	0.3	64.0
Necessary difference between inside and outside	3.8		4.8	
	Internal defects			
Oiled and bagged				
Inside	2.5	2.4	4.2	20.7
Outside	2.7	2.9	4.8	23.0
Control				
Inside	4.3	54.0	6.2	67.3
Outside	4.8	49.1	6.3	65.9
Necessary difference between inside and outside	2.4		3.0	

In the second experiment (Table V) there was likewise more spoilage, both external and internal, of central than of peripheral eggs when they were stored unbagged under the constant conditions of 70° F. and 90% relative humidity, and here again the effect of oiling and bagging was to eliminate this differential. It is noteworthy however that in the other part of this trial, in which humidity was maintained at a high level throughout both phases of the 20° temperature alternations, significantly less spoilage occurred in the outer rows of the oiled and bagged but not of the control eggs, i.e., just the reverse of the condition observed at constant temperature.

No significant effect of the position of tray in the crate on the development of spoilage was noted in the first experiment. In the second experiment, under the alternating temperature and high humidity conditions, there was more external mould in the middle trays of both oiled and bagged and control

eggs than in those located at either the top or bottom of the crate; there was also a tendency for the amount of internal spoilage to be greatest in the top trays and to diminish progressively towards the bottom of the cases. On the other hand, after storage at constant temperature and high humidity, position of the tray affected significantly only total internal defects, more spoilage developing at the bottom of the cases than at the top (the reverse of the trend observed under conditions of alternating temperature and high humidity). These results are shown in Table VI.

TABLE VI

AVERAGE PERCENTAGE OF SPOILAGE IN INSIDE AND OUTSIDE ROWS OF TRAYS (SECOND EXPERIMENT)

	Severe		Mild + severe	
	60 – 80° F., 90% R.H.	70° F., 90% R.H. continuously	60 – 80° F., 90% R.H.	70° F., 90% R.H. continuously
	External mould			
Oiled and bagged				
Inside	22.1	2.9	87.8	95.3
Outside	7.4	1.9	65.1	93.9
Control				
Inside	33.0	2.9	95.0	82.8
Outside	28.7	0.4	90.8	71.6
Necessary difference between inside and outside	13.3	5.0	6.8	4.1
	Internal defects			
Oiled and bagged				
Inside	12.9	1.1	32.3	8.3
Outside	4.9	1.9	15.3	8.4
Control				
Inside	84.9	48.0	96.6	73.8
Outside	75.7	40.6	95.4	71.6
Necessary difference between inside and outside	9.5	6.3	6.3	14.4

pH of White and White Thickness

In the first experiment it was possible to measure the pH and thickness (height) of the white of four eggs (two centrally and two peripherally located) from practically every tray of the oiled and bagged half-cases and of the controls stored under alternating conditions. Owing to almost invariable rupturing of the yolk-membrane, it was not possible to secure measurements

for the controls stored under constant conditions. Observations were thus secured on 215 oiled and bagged and 208 control eggs stored under alternating conditions, and on 213 oiled and bagged eggs stored under constant conditions.

Following storage under alternating conditions the average pH of the oiled and bagged eggs measured (8.63) was significantly lower than that of the controls (9.23). There was no difference between the replicate lots of eggs in this respect, and the manipulations incidental to the production of different degrees of shell contamination were also apparently without effect on the pH at the conclusion of storage.

White thickness was found to be much more sensitive to treatment and environment. After storage under alternating conditions the oiled and bagged eggs had an average white thickness one-third greater than that of the controls. Furthermore, this characteristic was affected by the initial shell treatment. The white of oiled and bagged eggs previously dipped in hydrogen peroxide was significantly thinner than that of those either contaminated or initially untreated; whilst of the controls (not oiled and bagged) the white of the peroxided eggs was significantly thinner than that of those with untreated shells, but did not differ significantly from those dipped in the contaminating suspension (Table VII). A comparison of the results for

TABLE VII

AVERAGE WHITE THICKNESS (IN.) OF EGGS HELD UNDER ALTERNATING CONDITIONS (FIRST EXPERIMENT)

Treatment	Shell condition			Average
	Normal	Reduced	Contaminated	
Oiled and bagged	0.103	0.087	0.106	0.099[1]
Untreated	0.075	0.059	0.063	0.066[1]
Average	0.089[2]	0.073[2]	0.083[2]	

[1] *Necessary difference = 0.008.*
[2] *Necessary difference = 0.009.*

TABLE VIII

AVERAGE WHITE THICKNESS (IN.) OF OILED AND BAGGED EGGS HELD UNDER ALTERNATING AND CONSTANT CONDITIONS (FIRST EXPERIMENT)

——	Shell condition			Average
	Normal	Reduced	Contaminated	
Alternating	0.103	0.087	0.106	0.099[1]
Constant	0.093	0.082	0.094	0.090[1]
Average	0.098[2]	0.084[2]	0.100[2]	

[1] *Necessary difference = 0.006.*
[2] *Necessary difference = 0.007.*

oiled and bagged eggs kept under alternating and constant conditions (Table VIII) indicated that the latter, if initially contaminated or untreated, had the thinner whites, whilst following both sets of storage conditions the peroxide dip resulted in a uniformly low value of this characteristic.

Discussion

The failure of the modifications of initial shell contamination imposed in the first of the present experiments to influence subsequent spoilage is noteworthy. From the data available it is impossible to determine whether this was due to the inefficacy of the methods of infection and disinfection adopted, or to the fact that spoilage is, within rather wide limits, dependent primarily upon environmental conditions rather than upon the quantity of inoculum originally present; the latter supposition however receives some support from the findings of Rosser (2) who, using a contaminating liquid containing from 50 to 100 times the number of organisms per unit volume in that employed by the present writers, was able to demonstrate a significant relation between initial contamination and subsequent storage. On the other hand, the adverse effect of peroxide dipping is in accordance with the observations of Rosser *et al.* (3) who noted that this treatment did not preserve either white thickness or yolk index. As oiling and bagging reduced the detrimental action appreciably (Table VII), it seems possible that this effect of the peroxide may have been partially offset by a greater retention of carbon dioxide by the oiled and bagged eggs, reflected in their lower pH.

The results of the two present experiments are in agreement with each other and with the earlier results of Reedman and Hopkins (1) in indicating a significant reduction of internal spoilage by oiling and bagging of the eggs stored under constant conditions of 70° F. and 90% relative humidity. Furthermore, although the degree of control (Tables I and III) fell short of that achieved in the preceding trial, when the incidence of all forms of internal defects was reduced to about 1%, it is also true that the percentage of total internal spoilage in untreated eggs in the two present trials, at 67 and 73%, was about 20% higher than the corresponding 51% occurring previously (1). Part of this increase is no doubt attributable to the extra week the present eggs were stored. External mould, on the other hand, was more pronounced in the preceding experiment than in the two present ones, but was also more amenable to control, even if allowance be made for some divergence of classification as "mild" and "severe" attributable to personal factors. It must be concluded therefore that the results of treatment may be dependent to some extent on the initial history of the eggs dealt with. Rosser (2) obtained his "market" eggs in February and their age was not known. Reedman and Hopkins (1) obtained their supplies in April from grading stations and the eggs were probably only 24 hr. old. In the present tests, in January and April, the ordinary run of grading station eggs, varying from one to seven days old, were used. This difference in age may account for the above mentioned

difference in the amount of spoilage. Since all tests were made when eggs are considered to be of the best quality, the season cannot be considered as a major factor.

In spite of the above incongruities however the discrepancies in spoilage, both external and internal, following storage at 65° to 75° F. and 95 to 70% relative humidity on the one hand, and at 60° to 80° F. and 90% on the other, are of an altogether greater order than those occurring in the two parallel series kept at 70° F. and 90% continuously, and accordingly, may reasonably be considered to result from the conditions of storage rather than from differential susceptibility of the eggs used on the two occasions. It is to be remarked that in all four series the mean storage temperature was approximately 70° F. In the first of the present experiments the alternating conditions were such that the dew point should never be reached and hence condensation should not occur. Owing to some uncontrolled variation, condensation was probably not altogether absent. In the second experiment the situation is complicated by the lag in the thermal and doubtless also in the humidity cycle within the crates relative to that in the surrounding atmosphere. It seems probable, however, that even after allowing for this the temperature of the eggs must have fallen below the dew point at some stage in the progression from 60° to 80° F. Condensation would therefore be expected to have more influence in this than the preceding set of alternating conditions.

The differential spoilage following storage at 70° F. and 90% relative humidity of eggs peripherally and centrally located in the trays, is in accordance with previous experience (1). At the conclusion of storage, condensate was observed in six of the nine cases held under these conditions in the first of the present experiments. It was noted that such condensate was most pronounced in the middle trays and on the eggs located in the centre of these but in view of the fact that this condition occurred in four lots of bagged as compared to three of unbagged eggs, it cannot be related directly to the incidence of spoilage. Whilst variations in spoilage within crates were again encountered in the second experiment, these did not parallel the trends previously observed, and the results as a whole justify only the general conclusion that unequal spoilage may occur, presumably as a consequence of intracrate temperature and humidity differentials such as those noted in the first experiment.

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